Deletion of chromosome bands 11q22-q23 in lymphoproliferative disorders and the genetics of mantle cell lymphoma

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1 LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

I Monni O, Zhu Y, Franssila K, Oinonen R, Höglund P, Elonen E, Joensuu H, Knuutila S. Molecular characterization of deletion at 11q22.1-23.3 in mantle cell lymphoma. **Br J Haematol** 1999, 104, 665-671.

II Zhu Y, Monni O, El-Rifai W, Siitonen SM, Vilpo L, Vilpo J, Knuutila S. Discontinuous deletions at 11q23 in B cell chronic lymphocytic leukemia. **Leukemia** 1999, 13, 708-712.

III Zhu Y, Monni O, Franssila K, Elonen E, Vilpo J, Joensuu H, Knuutila S. Deletions at 11q23 in different lymphoma subtypes. **Haematologica** 2000, 85, 908-912.

IV Zhu Y, Loukola A, Monni O, Kuokkanen K, Franssila K, Elonen E, Vilpo J, Joensuu H, Kere J, Aaltonen L, Knuutila S. *PPP2R1B* gene in chronic lymphocytic leukemias and mantle cell lymphomas. **Leuk Lymph** 2001, 41, 177-183.

V Zhu Y, Hollmén J, Oinonen R, Aalto Y, Elonen E, Kere J, Mannila H, Franssila K, Knuutila S. Gene expression profiling in mantle cell lymphoma and its blastoid variant. **Submitted**.

The publications are referred to by their Roman numerals in the text.

2 ABBREVIATIONS

BAC	bacteria artificial chromosome
CLL	chronic lymphocytic leukemia
cDNA	complementary deoxyribonucleic acid
CGH	comparative genomic hybridization
DAPI	4', 6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DLBCL	diffuse large B-cell lymphoma
DNA	deoxyribonucleic acid
dUTP	deoxyuridine triphosphate
FISH	fluorescence *in situ* hybridization
FITC	fluorescein-isothiocyanate
FL	follicular lymphoma
HL	Hodgkin lymphoma
kb	kilobase
kD	kilodalton
LOH	loss of heterozygosity
MALT	mucosa-associated lymphoid tissue
Mb	megabase
MCL	mantle cell lymphoma
mRNA	messenger ribonucleic acid
MSC	mechanically stretched chromosome
NHL	non-Hodgkin's lymphoma
NK	natural killer
p	chromosome short arm
PAC	P1 artificial chromosome
PAGE	polyacrylamide gel electrophoresis
PCA	principal component analysis
PCR	polymerase chain reaction
PGL	paraganglioma
q	chromosome long arm
RNA	ribonucleic acid
REAL	A revised European-American classification for lymphoid neoplasms
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SLL	small lymphocytic lymphoma
SSC	standard saline citrate
SSCP	single strand conformation polymorphism
TBE	Tris-borate/EDTA electrophoresis buffer
TRITC	tetra-rhodamine-isothiocyanate
WHO	World Health Organization
YAC	yeast artificial chromosome

3 ABSTRACT

Chromosome bands 11q22-q23 have been found frequently deleted in a number of solid tumors and lymphoproliferative disorders, suggesting the existence of tumor suppressor gene(s) in this area. This abnormality has been found in mantle cell lymphoma (MCL) and chronic lymphocytic leukemia (CLL) by comparative genomic hybridization studies (Karhu *et al.*, 1997; Monni *et al.*, 1998). By using the samples from MCL and CLL patients, we wanted to identify the minimal common region of deletion in 11q22-q23 and the candidate gene in the region. We also wanted to study the occurrence of the deletion in 11q22-q23 in different types of lymphoma. Because MCL is a relatively newly identified disease entity whose molecular background is not well known, it was also our aim to study the gene expression profiles of MCL and its blastoid variant.

Altogether we studied samples from 158 lymphoma or leukemia patients. One hundred and fifty-two samples were studied using fluorescence *in situ* hybridization (FISH) with YAC (yeast artificial chromosome) probes from 11q22.1-q23.3. Two critical regions were identified in 11q22-q23, one represented by YAC755b11 and the other by YAC785e12. The presence of the deletion in 11q23 is frequent in MCL, and the abnormality is present in a small fraction of CLL/SLL (small lymphocytic lymphoma) and diffuse large B-cell lymphoma cases. Our results showed the possible correlation of the deletion in 11q23 with the development of leukemia from localized lymphoma, and with the development of the Richter's syndrome. One candidate gene in 11q23, the *PPP2R1B* gene, was examined. The mutation analysis of this gene has suggested that the pathogenic role of the *PPP2R1B* gene in MCL and CLL is probably minor and it is not likely to be the target of the deletion in 11q23. In study V, we used the cDNA array technology to study the gene expression profiles of common and blastoid variant MCL. We studied 18 samples from MCL patients and identified marker genes for both common and blastoid variant MCL. We also created a disease subtype (common vs blastoid variant) classifier, which might be helpful in the differential diagnosis of MCL.

Some of the results obtained from this thesis work have already been utilized in the diagnosis of lymphomas.

4 INTRODUCTION

Non-Hodgkin's lymphoma (NHL) is a heterogeneous group of neoplasms of the immune system with distinct morphological, immunohistochemical, genetic and clinical features. To correctly classify and diagnose different subgroups of NHL is essential in order to treat the patients successfully.

One of the important criteria for lymphoma classification and diagnosis is the genetic abnormality associated with different lymphoma subgroups. It is widely acknowledged that cancer is a genetic disease, resulting from the accumulation of gene mutations. At the microscopic level, it is manifested by the structural abnormalities of the chromosomes. Research conducted over the past 30 years has shown that clonal chromosomal abnormalities in tumor cells are distributed non-randomly throughout the genome. Different neoplasms have distinctive chromosomal abnormality profiles involving different chromosomes, chromosomal arms, bands and sub-band regions. On the other hand, many abnormalities are associated with certain diseases or disease subgroups only (Heim & Mitelman, 1995).

In this thesis, we studied abnormalities of one particular chromosome part, namely the deletion in chromosome bands 11q22-q23, in several types of NHL, in particular mantle cell lymphoma and chronic lymphocytic leukemia. Our intention was to determine the molecular background of this abnormality and to investigate any clinical correlation it might have. We further studied mantle cell lymphoma on the gene expression level in order to understand more about the pathogenesis of this disease.

Modern genetic research is characterized by the technical advancement. Various novel techniques and methods enable research on both the single-gene and the genome level. Results obtained in recent years have greatly improved our knowledge of the diseases. Today, the use of many of the techniques, such as the chromosome banding analysis and the fluorescence *in situ* hybridization analysis, is considered an important part in disease diagnosis and prognosis.

5 REVIEW OF THE LITERATURE

5.1 Lymphocytic malignancies

5.1.1 Classification of lymphoma

Non-Hodgkin's Lymphoma (NHL) is a heterogeneous group of neoplasms of the immune system. The classification of NHL is not a simple task, because many different cell types are involved. Moreover, the neoplasms can originate in virtually any organ, and patients with some types of lymphoma can develop leukemia. It is of great importance to correctly classify and diagnose the different subgroups of NHL in order to treat these diseases successfully. A common system of classification helps clinicians and pathologists all over the world to communicate and exchange information. Since 1925, at least 25 classification systems have been developed for NHL. The ones that can be regarded as milestones are the Rappaport classification (Rappaport, 1966), the Kiel classification (Lennert, 1978; Stansfeld *et al.*, 1988), the Lukes and Collins classification (Lukes & Collins, 1974), the Work Formulation for Clinical Usage (Non-Hodgkin's Lymphoma Pathologic Classification Project, 1982), the Revised European-American Lymphoma (REAL) classification (Harris *et al.*, 1994) and the WHO (World Health Organization) classification (Jaffe *et al.*, 2001). The history of NHL classification clearly reflects our increasing understanding of the nature of different subgroups of NHL. The focus of the classification criteria has gradually shifted from pure morphology or clinical behavior to include neoplastic cell morphology, immunophenotype, genetic background, normal cell counterpart and clinical features. In other words, all the current information is now used collectively to define the disease entities. The development of NHL classification also reflects the amazing technical and instrumental advances in the field over the past 40 years. New technologies allow more thorough study of the diseases, leading to a more profound understanding of them [for review see (Isaacson, 2000)].

The classification systems most widely in use now are the REAL classification and its updated version - the WHO classification (Table 1), which were both jointly drawn up by experts from Europe and the United States. In both classification systems, lymphomas are classified based on five properties: morphology, immunophenotype, genotype, normal cell counterpart and clinical features. In addition to malignancies originated from B and T cells, Hodgkin's lymphoma and

malignancies originated from natural killer (NK) cells are also included. The REAL classification has been proved to be highly practical and reproducible (Lymphoma Classification Project, 1997). Many specific genetic abnormalities were associated with particular lymphoma subtypes identified by the REAL/WHO classification (Jaffe *et al.*, 2001), proving the accuracy of the classification at the molecular level. The experience gained from developing the REAL and WHO classification could also be used in the classification of other types of cancer and will be important for the future development of lymphoma classification (Harris *et al.*, 2000).

It is clear from the history of NHL classification that there has been an evolution in the process. With the development of new technologies and the acquisition of new data, new disease entities may be identified, and uncertainties will be clarified. This is already taking place. For example, new subtypes of diffuse large B-cell lymphoma (DLBCL) have been identified using the microarray technology (Alizadeh *et al.*, 2000) and new subtypes of chronic lymphocytic leukemia (CLL) have been recognized by analyses of the Ig V gene mutation and the expression of CD38 (Damle *et al.*, 1999; Hamblin *et al.*, 1999). There is also more and more information being gathered on the correlation between genetic markers and patients' clinical behavior. It is possible that, in the future new approaches based on genetics and molecular biology may play an important role in NHL classification.

Table 1. The World Health Organization classification of lymphoid malignancies (Jaffe *et al.*, 2001)

B-cell neoplasms
Precursor B lymphoblastic leukemia/lymphoma
Chronic lymphocytic leukemia/small lymphocytic lymphoma
B-cell prolymphocytic leukemia
Lymphoplasmacytic lymphoma
Splenic marginal zone lymphoma
Hairy cell leukemia
Plasma cell myeloma
Monoclonal gammopathy of undetermined significance
Solitary plasmacytoma of bone
Extraosseous plasmacytoma
Primary amyloidosis
Heavy chain diseases
Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT-lymphoma)
Nodal marginal zone B-cell lymphoma
Follicular lymphoma
Mantle cell lymphoma
Diffuse large B-cell lymphoma
Mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
Primary effusion lymphoma
Burkitt lymphoma/leukemia
T-cell and NK-cell neoplasms
Precursor T lymphoblastic leukemia/lymphoma
T-cell prolymphocytic leukemia
T-cell large granular lymphocytic leukemia
Aggressive NK cell leukemia
Adult T-cell leukemia/lymphoma
Mycosis fungoides
Sézary syndrome
Primary cutaneous anaplastic large cell lymphoma
Lymphomatoid papulosis
Extranodal NK/T cell lymphoma, nasal type
Enteropathy-type T-cell lymphoma
Subcutaneous panniculitis-like T-cell lymphoma
Angioimmunoblastic T-cell lymphoma
Peripheral T-cell lymphoma, unspecified
Anaplastic large cell lymphoma
Blastic NK cell lymphoma
Hodgkin lymphoma
Nodular lymphocyte predominant Hodgkin lymphoma
Classical Hodgkin lymphoma
Nodular sclerosis classical Hodgkin lymphoma
Mixed cellularity classical Hodgkin lymphoma
Lymphocyte-rich classical Hodgkin lymphoma
Lymphocyte-depleted classical Hodgkin lymphoma

5.1.2 Mantle cell lymphoma

Mantle cell lymphoma (MCL) is a malignant non-Hodgkin's lymphoma of B cell lineage. The normal cell counterpart of the malignant cell of MCL is the immature CD5+ virgin B cell in the mantle zone of lymphoid follicles (Harris *et al.*, 1994). MCL has been described variously since its initial recognition in the mid-1970s, such as lymphocytic lymphoma of intermediate differentiation (Berard & Dorfman, 1974), centrocytic lymphoma (Lennert & Feller, 1990) and mantle zone lymphoma (Weisenburger *et al.*, 1981). It was later shown (Banks *et al.*, 1992) that these lymphomas were in fact one disease entity and given the name mantle cell lymphoma. MCL accounts for 2.5% to 4.0% of all NHL cases in the United States, and 7% to 9% in Europe (Weisenburger & Armitage, 1996). MCL patients are characterized by advanced age, male predominance, presentation at advanced stages, and frequent involvement of bone marrow, peripheral blood and other external sites (Fisher *et al.*, 1995; Norton *et al.*, 1995; Teodorovic *et al.*, 1995; Velders *et al.*, 1996; Argatoff *et al.*, 1997). The median survival of MCL patients is only 3 to 4 years in most large-scale series, even after combination chemotherapies for aggressive lymphomas (Oinonen *et al.*, 1998), so that MCL is regarded as an incurable disease.

The common form of MCL is characterized by small to medium-sized lymphocytes with scant cytoplasm. The nuclei usually have slightly irregular-contours with dispersed chromatin and inconspicuous nucleoli (Harris *et al.*, 1994). As in chronic lymphocytic leukemia (CLL), CD5 and pan B-cell antigens (CD19, CD20, CD22, and CD24) are usually co-expressed on MCL neoplastic cells. The feature distinguishing MCL cells from CLL cells is the expression of CD23, which is positive for CLL and usually negative for MCL. CD20 and immunoglobulin light chain expression is usually strong in MCL and weak in CLL, which is also a useful distinction. However, exceptions have been identified in both diseases. In addition to the common form of MCL, a so-called large cell or blastoid variant of the disease has been identified (Jaffe *et al.*, 1987; Fisher *et al.*, 1995; Ott *et al.*, 1997). Greiner et al. described three subtypes of the variant form of MCL: blastic; anaplastic; and centrocytoid-centroblastic (Greiner *et al.*, 1996), whereas Ott et al. identified only two types of variant (lymphoblastoid and pleomorphic variant) based on morphology (Ott *et al.*, 1997). Regardless of the morphological sub-classification, the feature distinguishing blastoid variant MCL from the common MCL is the high mitotic rate

(Jaffe *et al.*, 1987; Ott *et al.*, 1994; Fisher *et al.*, 1995). Around 30% of MCL patients develop the more aggressive blastoid variants that progress more rapidly and shorten survival (Norton *et al.*, 1995; Greiner *et al.*, 1996).

5.1.3 Chronic lymphocytic leukemia

Historically, chronic lymphocytic leukemia (CLL) has two subtypes: B-cell chronic lymphocytic leukemia (B-CLL) and T-cell chronic lymphocytic leukemia (T-CLL). About 97% of CLL cases are B-CLL (Bennett *et al.*, 1989). In the WHO lymphoma classification, however, CLL is recognized as a B-cell neoplasm only. The disease previously known as T-CLL is now recognized as T-cell prolymphocytic leukemia or large granular lymphocyte leukemia (Jaffe *et al.*, 2001). Here we will use the term CLL as defined by the WHO lymphoma classification. Small lymphocytic lymphoma (SLL) cases have the same tissue morphology and immunophenotype as CLL cases. CLL involves primarily bone marrow and peripheral blood, while SLL is usually non-leukemic, although exceptions exist in both diseases. Therefore, CLL and SLL are considered one disease entity (Harris *et al.*, 1994; Jaffe *et al.*, 2001).

CLL is the most common leukemia in adults in western countries and accounts for one fourth of all leukemia cases (Rozman & Monserrat, 1995). CLL affects mostly elderly patients, only 10% to 15% of patients are younger than 50 years at the time of diagnosis. There is a gender bias so that the disease affects more men than women (ratio approximately 2:1). Although the cause of the disease is not completely understood, one suggestion is the failure of malignant cells to undergo apoptosis. It has been shown that CLL patients accumulate mature monoclonal CD5+ lymphocytes and that these cells are arrested in the G_0/G_1 phase of the cell cycle (Bannerji & Byrd, 2000).

The immunophenotype of typical CLL malignant cells are: weak IgM or IgM and IgD, CD5+, CD19+, weak CD20, weak CD22, CD79a+, CD23+, CD43+, weak CD11c, CD10-, cyclin D1-, and FMC7 and CD79b negative or weakly expressed. Matutes et al. (1994) developed a scoring system for differential diagnosis of CLL from other types of B-cell leukemia based on the immunophenotype. Typical CLL case has a score of five, which means CD22-, CD23+, CD5+, FMC7- and undetectable or weak Igκ/λ (Matutes *et al.*, 1994).

The normal cell counterpart of the leukemia cells in CLL was originally thought to be the virgin B cell. However, recent studies have shown that the normal cell counterpart is the virgin B cell in only about 50% of cases of CLL, and that it is the memory B cell in the other cases (Damle *et al.*, 1999; Hamblin *et al.*, 1999). The same studies have shown that the disease is less malignant in those cases where the normal cell counterpart is the memory B cell, than when the normal cell counterpart is the virgin B cell.

CLL is usually considered an incurable disease with currently available therapies. The clinical course of CLL is in general indolent, but varies a lot among patients. Some patients can live asymptomatically for many years, while others die within five years after diagnosis. There are two staging systems – Rai and Binet – in use for predicting the survival. The Rai staging system defines five stages (0-IV) with increasingly worse prognosis: lymphocytosis alone (stage 0); lymphocytosis, lymphadenopathy (stage I); lymphocytosis, spleen or liver enlargement or both (stage II); lymphocytosis, anemia with hemoglobin less than 11.0 g/dl (stage III); lymphocytosis, thrombocytopenia with platelet count less than 100,000 /mm^3 (stage IV) (Rai *et al.*, 1975). The Binet staging system defines three stages (A-C) with increasingly worse prognosis: no anemia, no thrombocytopenia, less than three areas enlarged (A); no anemia, no thrombocytopenia, three or more areas enlarged (B); anemia with hemoglobin less than 10.0 g/dl, or thrombocytopenia with platelet count less than 100,000/mm^3, or both (C) (Binet *et al.*, 1981).

Around 3.5% of CLL cases transform to high-grade lymphoma, a process referred to as Richter's syndrome (Richter, 1929; Foucar, 1992). Around 3% of the Richter's syndrome cases transform to diffuse large B-cell lymphoma, and the rest transform to lymphomas resembling Hodgkin's lymphoma.

5.2 11q and genetic aberrations in non-Hodgkin's lymphoma

5.2.1 Genes and abnormalities in 11q

Chromosome 11 represents about 4.8% of the relative autosome length, is estimated to be 144 Mb in size, and has 997 genes assigned to it. The long arm of chromosome 11, 11q, is estimated at around 90 Mb and has 673 genes assigned to it (http://www.ncbi.nlm.nih.gov). Genes are non-uniformly distributed along 11q, clustering mostly in three regions, 11q13, 11q22-q23 and 11q24. Proto-oncogenes *EMS1*, *FGF3* (*INT2*), *FGF4* (*HST*), *CCND1* (*PRAD1*), *BCL1* and *GSTP1* are located in 11q13. The candidate gene for MEN1 (multiple endocrine neoplasia type 1) has been localized to 11q13.1 (Chandrasekharappa *et al.*, 1997), as has *PGL2*, one of the two paraganglioma (PGL) candidate genes located on chromosome 11q (Mariman *et al.*, 1993). The translocation t(11;22)(q24;q12), present in about 90% of cases of Ewing's sarcoma and neuroepithelioma (Turc *et al.*, 1984), involves the oncogene *FLI1* in 11q24 (Hromas *et al.*, 1993). The gene encoding the protein kinase CHK1, a checkpoint protein for the G2 to M phase transition, is located in 11q24 (Sanchez *et al.*, 1997). In 11q24 there is also the *ETS1* gene, which is homologous to the viral *ets* oncogene of the E26 virus, and acts as a transcription factor in regulating cell proliferation, differentiation, lymphoid cell development, angiogenesis and apoptosis (Li *et al.*, 1999).

Chromosome bands 11q22-q23 are frequently involved in translocations and deletions in a variety of neoplasms. Figure 1 shows the schematic integrated physical map of this region (Arai *et al.*, 1996). The most common chromosomal translocations in this region are those involving the *MLL* gene, of which more than 40 different ones have been described and 19 partner genes have been cloned (Osaka *et al.*, 1999; Schreiner *et al.*, 1999). These translocations are present in about 5% of patients with acute myeloid leukemia, and up to 10% of patients with acute lymphocytic leukemia (Kaneko *et al.*, 1986). In addition, they are seen in up to 80% of cases of infant acute myeloid leukemia and acute lymphocytic leukemia (Heerema *et al.*, 1994). Around 85% of patients with topoisomerase II inhibitor-related secondary leukemia have these translocations (Pedersen-Bjergaard & Rowley, 1994).

Cytogenetic and loss of heterozygosity (LOH) analyses have shown that chromosome bands 11q22-q23 are frequently deleted in a number of solid tumors

(breast, cervical, ovarian, gastric, lung, bladder, prostate, nasopharyngeal, squamous and colorectal carcinomas; malignant melanomas and intracerebral neoplasms) (Foulkes *et al.*, 1993; Keldysh *et al.*, 1993; Carter *et al.*, 1994; Hampton *et al.*, 1994a; Hampton *et al.*, 1994b; Bethwaite *et al.*, 1995; Gabra *et al.*, 1995; Herbst *et al.*, 1995; Iizuka *et al.*, 1995; Koreth *et al.*, 1995; Negrini *et al.*, 1995; Rasio *et al.*, 1995; Shaw & Knowles, 1995; Winqvist *et al.*, 1995; Baffa *et al.*, 1996; Blaeker *et al.*, 1996; Davis *et al.*, 1996; Gabra *et al.*, 1996; Hui *et al.*, 1996; Tomlinson & Bodmer, 1996; Uzawa *et al.*, 1996; Dahiya *et al.*, 1997; Koreth *et al.*, 1997), as well as in lymphoproliferative disorders (Heim & Mitelman, 1995). These results strongly suggest the presence of tumor suppressor gene(s) in this region. Functional evidence for the existence of tumor suppressor gene(s) in 11q has been provided by microcell fusion experiments involving the transfer of normal chromosome 11 or part of it into malignant melanoma, breast, lung, cervical and ovarian cancer cell lines. Transfer of the whole chromosome 11, the chromosome bands 11q13-q23 or their fragments suppressed the *in vitro* growth of the cells (Gioeli *et al.*, 1997), and the *in vivo* tumorigenesis (Negrini *et al.*, 1994; Zenklusen *et al.*, 1995; Robertson *et al.*, 1996; Murakami *et al.*, 1998) and metastatic potentials (Phillips *et al.*, 1996) of the cells in nude mice. While the length of the deleted regions varies between tumor types, two minimum common regions of deletion (common to three or more tumor types) were found in 11q22.3-q23.1 and 11q23.2-q23.3.

The minimal common region of deletion in 11q22.3-q23.1 has been found to be 2-3 Mb in size, and contain the *ATM* gene and a region represented by YAC755b11 (Stilgenbauer *et al.*, 1996; Koreth *et al.*, 1999). Other studies showed that the minimal critical region was only the region represented by YAC755b11 (1.6 Mb in size) (Monni *et al.*, 1999; Zhu *et al.*, 1999). *ATM* encodes a serine-threonine kinase belonging to a protein family related to phosphoinositide kinases which includes ATR, Mec1, Tel1 and Rad53 (Elledge, 1996). ATM detects DNA damages and activates p53, Chk2 and Mdm2 to promote apoptosis or cell cycle arrest. ATM also activates c-Abl in response to stress signals. In addition, ATM is required for DNA repair and insufficient DNA repair will lead to genomic instability (Baskaran *et al.*, 1997; Shafman *et al.*, 1997; Banin *et al.*, 1998; Canman *et al.*, 1998; Matsuoka *et al.*, 1998; Brown *et al.*, 1999; Johnson *et al.*, 1999). Inactivation of the *ATM* gene and the corresponding loss of ATM protein function result in ataxia telangiectasia, a

disorder characterized by atrophy of the cerebellum and thymus, immunodeficiency, premature aging, predisposition to cancer, and sensitivity to ionizing radiation (Savitsky *et al.*, 1995). Mutations of the *ATM* gene have been found in CLL, MCL and T-cell prolymphocytic leukemia cases (Stilgenbauer *et al.*, 1997; Bullrich *et al.*, 1999; Schaffner *et al.*, 1999; Stilgenbauer *et al.*, 1999; Schaffner *et al.*, 2000; Camacho *et al.*, 2002). The *PPP2R1B* gene is located very close to the region represented by YAC755b11. PP2A is a regulatory enzyme that negatively regulates the MAPK cascade and has been linked to carcinogenesis (Hunter, 1995). The *PPP2R1B* gene has 15 exons and encodes the β isoform of the structural/regulatory A subunit of the *PP2A* gene. It was found to be mutated in human lung and colon cancers, and identified as a putative tumor suppressor gene (Wang *et al.*, 1998). But no mutation was found in hereditary PGL (Baysal *et al.*, 1998), and rarely in ovarian carcinomas (Campbell & Manolitsas, 1999; Wu *et al.*, 1999), parathyroid hyperplasia and adenomas (Hemmer *et al.*, 2002), CLL or MCL (Zhu *et al.*, 2001). Therefore, its role as a tumor suppressor gene remains to be confirmed.

The 11q23.2-q23.3 region was found frequently deleted in cutaneous malignant melanoma (Herbst *et al.*, 1999), ovarian cancer (Launonen *et al.*, 1998), cervical carcinoma (Mugica-Van Herckenrode *et al.*, 1999), lung cancer (Wang *et al.*, 1999), breast cancer (Launonen *et al.*, 1999), CLL (Zhu *et al.*, 1999) and MCL (Monni *et al.*, 1999). A tumor suppressor gene, *TSLC1* (for tumor suppressor gene in lung cancer-1), was recently identified in non-small-cell-lung cancer. This gene is located in a 100 kb area in the center of the region represented by YAC939b12 (Murakami *et al.*, 1998; Kuramochi *et al.*, 2001). 11q23.2 is also the region where the gene responsible for hereditary PGL is located. PGL is a rare disorder characterized by the development of mostly benign, highly vascular, slow-growing tumors in the head and neck. The succinate-ubiquinone oxidoreductase subunit D gene (*SDHD*) was recently identified as the hypothesized gene *PGL1*. *SDHD* is a critical component of the oxygen-sensing system of paragangliomic tissue. The loss of its function may lead to chronic hypoxic stimulation and cellular proliferation (Baysal *et al.*, 2000). It will be interesting to examine the roles of these two genes in other types of neoplasms.

A retinoid-induced class II tumor suppressor / growth regulatory gene was found to be involved in CLL and located in 11q23 (DiSepio *et al.*, 1998). Its exact location and role in other types of cancer deserve further investigation.

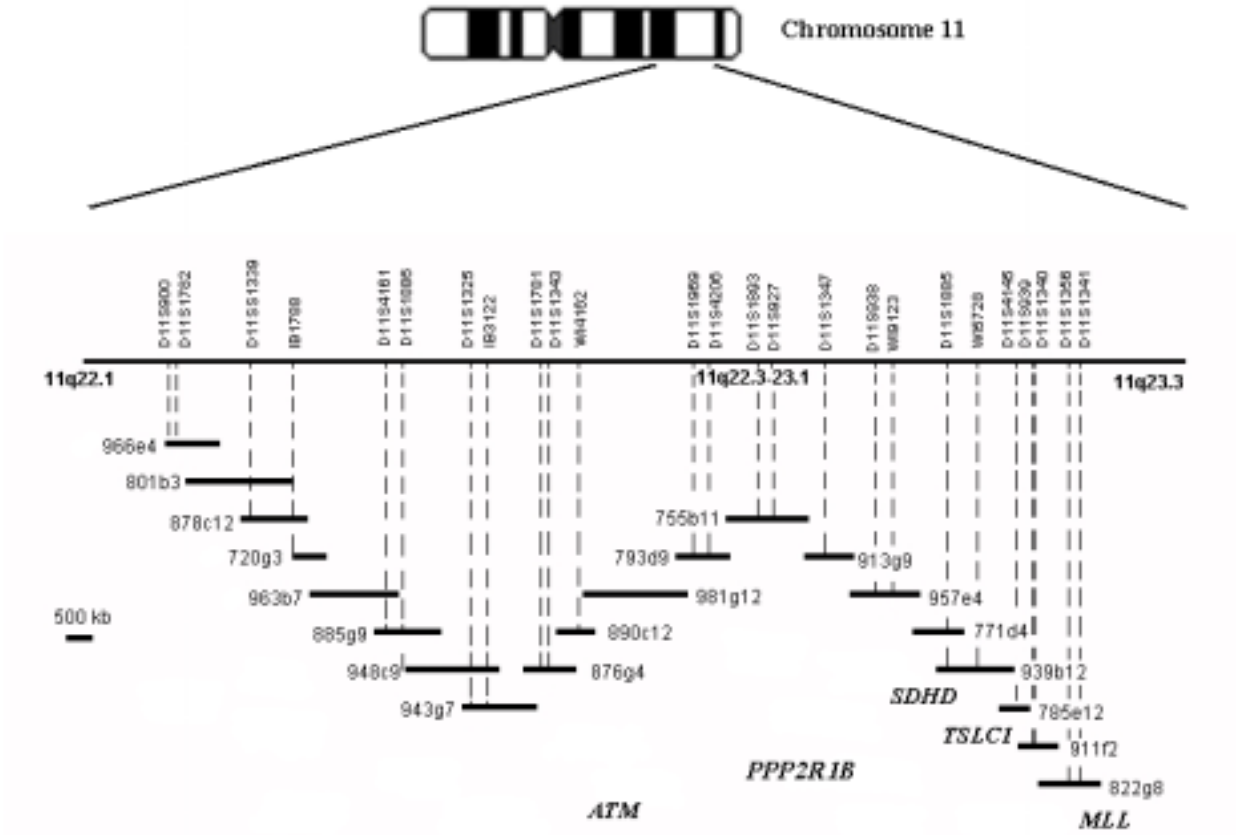

Figure 1. Schematic physical map of the region 11q22.1-q23.3 and some important genes (below) [a modification of (Arai et al. 1996)].

5.2.2 Chromosomal abnormalities in non-Hodgkin's lymphoma

Tumor cells exhibit clonal chromosomal abnormalities that are non-randomly distributed throughout the genome. It has been shown that many types of cancer have distinctive chromosomal aberration profiles, and some chromosome aberrations are unique to certain types of cancer or cancer subgroups (Heim & Mitelman, 1995).

Many NHL subgroups have characteristic chromosomal translocations. For example, t(14;18)(q32;q21) is found in 70% to 90% of follicular lymphoma cases, resulting in deregulation of the oncogene *BCL2* (Tsujimoto *et al.*, 1985). MCL is characterized by t(11;14)(q13;q32), which is found in 50% to 70% of cases and involves the oncogene *BCL1* (Weisenburger & Armitage, 1996). t(8;14)(q24;q32) is found in 75% to 85% of Burkitt's lymphoma and involves the *CMYC* gene (Dalla-Favera *et al.*, 1982). The translocation t(9;22)(q34;q11) creates an oncogenic fusion gene *BCR-ABL*, and is found in 85% of cases of chronic myeloid leukemia and 15%-20% of cases of acute lymphocytic leukemia (Heim & Mitelman, 1995).

Trisomies of chromosomes 3, 7, 12 and 18 have been found in NHL with varying frequencies (Heim & Mitelman, 1995). Other chromosomal amplifications detectable using cytogenetic analysis techniques, such as double minute chromosomes and homogeneously staining regions, are rarely detected in NHL (Ben-Yehuda *et al.*, 1994). Comparative Genomic Hybridization (CGH) studies have revealed several highly amplified regions in NHL, and oncogenes in these regions were also found to be amplified [reviewed by (Knuutila *et al.*, 1998)].

The most common chromosome deletion sites in NHL include 6q, 11q, 13q and 14q (Heim & Mitelman, 1995). In addition, CGH studies have found frequent chromosome deletions in 1p, 8p, 9p, 12p, 12q and 17p in NHL (Knuutila *et al.*, 1999). The characteristic chromosomal aberrations in NHL detected by chromosome banding analysis are summarized in Table 2.

Recurrent observation of deletion in a chromosome in tumor samples by cytogenetic analysis often indicates the existence of a putative tumor suppressor gene. Tumor suppressor genes encode proteins that function in cell growth regulatory or differentiation pathways, and loss of their function induces cells to develop malignant phenotypes (Vogelstein & Kinzler, 1998). The loss of function of tumor suppressor genes usually follows the "two-hit" model originally proposed to explain the

development of retinoblastoma by Alfred Knudson (Knudson, 1971). According to this model, two mutagenic events (hits) are required for the tumor suppressor genes to stop functioning. In familial cancers, the first hit is present in the germline and the second hit comes as a somatic mutation. In sporadic cancers, both mutagenic events are somatic. Inactivation of a tumor suppressor gene often occurs through the deletion of one allele and mutation of the other. In addition to cytogenetic analysis, tumor suppressor genes can also be identified through DNA linkage analyses attempting to locate genes involved in an inherited predisposition to cancer, and through LOH or allelic loss studies (Vogelstein & Kinzler, 1998). Examples of known and putative tumor suppressor genes have been summarized by Knuutila et al. (1999).

Table 2. Characteristic chromosomal aberrations in malignant lymphoma and lymphocytic leukemia [a modification of (Heim & Mitelman, 1995)]

Rearrangement	**Gene(s) involved**	**Types of lymphoma / Leukemia**
1p and 1q rearrangements	?	Variable B- or T-cell NHL, Hodgkin lymphoma, multiple myeloma, plasma cell leukemia
t(1;19)(q23;p13)	*E2A;PBX1*	Acute lymphocytic leukemia
t(2;3)(p12;q27)	*IGK;LAZ3/BCL6*	Diffuse large B-cell or follicular lymphoma
t(2;5)(p23;q35)	*ALK;NPM*	Ki-1 lymphoma
t(2;8)(p12;q24)	*IGK;MYC*	Burkitt lymphoma, acute lymphocytic leukemia
t(2;18)(p12;q21)	*IGK;FVT1*	Follicular lymphoma
+3	?	Variable B- or T-cell NHL
3q rearrangements	?	Variable B- or T-cell NHL, Hodgkin lymphoma
t(3;14)(q27;q32)	*LAZ3/BCL6;IGH*	Diffuse large B-cell or follicular lymphoma
t(3;22)(q27;q11)	*LAZ3/BCL6;IGL*	Diffuse large B-cell or follicular lymphoma
6p rearrangements	?	T-cell NHL
del(6q)	?	Variable, mostly B-cell NHL, Hodgkin lymphoma, acute lymphocytic leukemia, chronic lymphocytic leukemia, hairy cell leukemia, T-cell prolymphocytic leukemia
+7	?	Variable B- or T-cell NHL
7q rearrangements	?	Hodgkin lymphoma

t(8;14)(q24;q32)	*MYC;IGH*	Burkitt lymphoma, acute lymphocytic leukemia
t(8;22)(q24;q11)	*MYC;IGL*	Burkitt lymphoma, acute lymphocytic leukemia
9q rearrangements	?	Variable B- or T-cell NHL
t(9;14)(p13;q32)	*PAX5;IGH*	Small lymphocytic lymphoma
t(9;22)(q34;q11)	*BCR;ABL*	Acute lymphocytic leukemia
t(10;14)(q24;q11)	*HOX11;TCRD*	T-cell acute lymphocytic leukemia
t(10;14)(q24;q32)	*LYT10;IGH*	Variable B-cell NHL
del(11q)	?	Chronic lymphocytic leukemia, mantle cell lymphoma
t(11;14)(q13;q32)	*BCL1/PRAD1;IGH*	Mantle cell lymphoma, chronic lymphocytic leukemia, multiple myeloma, plasma cell leukemia
t(11;18)(q21;q21)	?	Small lymphocytic lymphoma, mucosa-associated lymphoid tissue (MALT) lymphoma
rearrangement of 11q23	*MLL*; multiple fusion genes	Acute lymphocytic leukemia
+12	?	Small lymphocytic or diffuse large B-cell lymphoma, chronic lymphocytic leukemia, B-cell prolymphocytic leukemia
12p rearrangements	?	Hodgkin lymphoma
13p rearrangements	?	Hodgkin lymphoma
del/+(13q)	?	Chronic lymphocytic leukemia
14q+	?	Chronic lymphocytic leukemia, B-cell prolymphocytic leukemia, hairy cell leukemia, multiple myeloma, plasma cell leukemia
14q11 rearrangements	*TCRA;TCRD*	T-cell NHL, T-cell prolymphocytic leukemia
del(14q)	?	Variable B- or T-cell NHL
14q32 rearrangements	*IGH*	Variable B-cell NHL, Hodgkin lymphoma
t(14;18)(q32;q21)	*IGH;BCL2*	Follicular or diffuse large B-cell lymphoma
del(17p)	?	Chronic lymphocytic leukemia
+18	?	Variable B- or T-cell NHL
t(18;22)(q21;q11)	*BCL2;IGL*	Follicular lymphoma
-X/+X/-Y	?	Variable B- or T-cell NHL

5.2.3 Genetic aberrations in mantle cell lymphoma

Over-expression of cyclin D1 is a characteristic feature of MCL. A recent study by Yatabe et al. showed that 85% of MCL cases were cyclin D1 positive. They suggested that the cyclin D1 positive and negative cases might actually represent different disease entities, with the cyclin D1 positive cases representing typical MCL, while the cyclin D1 negative cases could be better described as "cyclin D1-negative MCL-like B-cell lymphoma" cases (Yatabe *et al.*, 2000). The over-expression of cyclin D1 in more than 70% of the cases are caused by the t(11;14) chromosomal translocation (Williams *et al.*, 1992). MCL was also found to be characterized by the inactivation of the *ATM* gene (Stilgenbauer *et al.*, 1999; Schaffner *et al.*, 2000; Camacho *et al.*, 2002). *P53* mutations were found in some MCL cases, and it was suggested that these mutations were correlated with a poor prognosis (Greiner *et al.*, 1996). In the blastoid variants of MCL, abnormalities of the cyclin-dependent kinase inhibitor genes $P16^{INK4a}$ and $P21^{Waf1}$, were also found (Pinyol *et al.*, 1997; Pinyol *et al.*, 1998).

CGH studies have shown that MCL cases have a rather different pattern of chromosomal alterations compared to ones detected in other types of NHL. The most common abnormalities were recurrent gains in 3q, 7p, 8q, 12q, 15q, 18q and 9q34, and losses in 1p, 6q, 9p, 10p14-p15, 11q14-q23, 13 and 17p (Monni *et al.*, 1998; Beà *et al.*, 1999). Blastoid variant MCL was found to have increased number of chromosomal imbalances, high-level DNA amplifications, and a tendency to be tetraploid (Ott *et al.*, 1997; Beà *et al.*, 1999).

5.2.4 Genetic aberrations in chronic lymphocytic leukemia

CLL has been reported in ataxia telangiectasia families (Swift *et al.*, 1987), although no evidence of linkage between CLL and the *ATM* gene was found (Bevan *et al.*, 1999). Somatic deletion or point mutation affecting both alleles of the *ATM* gene have been reported (Schaffner *et al.*, 1999). Germ line mutations of the *ATM* gene have also been found in one third of CLL patients (Bullrich *et al.*, 1999), and total or partial inactivation of ATM protein in up to 40% of patients (Stankovic *et al.*, 1999). Patients with decreased expression of the ATM protein have more aggressive disease and shorter survival (Starostik *et al.*, 1998). In about 95% of cases of CLL there is increased expression of the *BCL2* oncogene, due to DNA hypomethylation in the *BCL2* promoter region (Hanada *et al.*, 1993). Mutations of the *P53* tumor suppressor

gene were seen in 15% of patients and patients with *P53* mutations had more aggressive disease (Cordone *et al.*, 1998). Some CLL patients have leukemic cells showing high-level expression of $P27^{Kip1}$, whose protein product normally decreases as a cell progresses into S phase. Such patients may have shorter blood lymphocyte doubling time and shorter survival than the average CLL patient (Vrhovac *et al.*, 1998).

Chromosome banding analysis reveals clonal chromosome aberrations in around 40-50% of CLL patients (Juliusson & Gahrton, 1990; Juliusson *et al.*, 1991). This technique is likely to underestimate the prevalence of clonal chromosome aberrations because the leukemic cells have low spontaneous mitotic activity and respond poorly to mitogenic stimulation (Autio *et al.*, 1987). When mitogenic stimulation is optimized, clonal chromosome aberrations were found in up to 79% of CLL patients (Larramendy *et al.*, 1998). CGH and interphase fluorescence *in situ* hybridization (FISH) techniques have also greatly facilitated the detection of chromosomal aberrations in CLL.

The most frequent chromosomal aberrations were found to be deletions in 13q, 11q, 6q and 17p, and trisomy 12 (Döhner *et al.*, 1999). The aberration in 13q mainly involves the chromosome band 13q14, where the retinoblastoma tumor suppressor gene (*RB1*) resides. It has been speculated that *RB1* could have a pathogenic role in CLL, but the results so far have been contradictory. The pathogenic gene is thought more likely to be located distal to *RB1* and efforts at positional cloning have been made (Liu *et al.*, 1997; Corcoran *et al.*, 1998; Stilgenbauer *et al.*, 1998). Patients with the 11q deletion were shown to represent a subset of CLL characterized by extensive nodal involvement and poor prognosis (Döhner *et al.*, 1997). Recently, cDNA microarray analysis has revealed a distinctive gene expression profile for CLL patients with the 11q23 deletion (Aalto *et al.*, 2001). Trisomy 12 was the most frequent chromosomal aberration found using chromosome banding analysis, with the frequency ranging from approximately 7% to more than 25% (Juliusson & Gahrton, 1990). Trisomy 12 was shown to be related to atypical morphology and poor prognosis (Escidoer *et al.*, 1993; Que *et al.*, 1993; Criel *et al.*, 1994; Matutes *et al.*, 1996). Patients with deletions in chromosome 17 usually have the poorest prognosis and this aberration is the only one of independent prognostic value (Bentz *et al.*, 1999; Döhner *et al.*, 2000).

5.3 Recent technological advances in molecular cytogenetics

5.3.1 Chromosome banding analysis

The chromosome banding technique was first introduced in 1968 (Caspersson *et al.*, 1968), and has ever since played a central role in genetic research and clinical applications (Heim & Mitelman, 1995). However, cytogenetic analysis is sometimes very problematic due to the difficulties in obtaining high quality metaphase or prometaphase spreads of dividing neoplastic cells, in order to fix and stain the chromosomes properly before the microscopic examination, especially in solid tumors. Consequently, chromosome banding analysis data for neoplasms are the most abundant for leukemias (63%), followed by solid tumors (27%) and lymphomas (10%) (Mitelman, 1994). In addition, chromosome structural rearrangement involving segments smaller than a band (around 10 Mb) cannot be detected using the banding analysis (Heim & Mitelman, 1995, page 24).

5.3.2 FISH

The FISH analysis utilizes the specific base pairing of two complementary nucleic acid sequences, one from the probe and the other from the target that is fixed on a microscopic slide. The interaction between the probe and the target can be visualized under the microscope through direct or indirect fluorescence labeling of the probe. The FISH technique is widely used in chromosome structure studies and for genome mapping.

Probes

The probes used in FISH can be broadly assigned to two groups: the probes for repetitive sequences and the locus-specific probes.

Examples of probes for repetitive sequences are the centromere specific probes. Chromosome centromere specific probes have been developed for all human chromosomes except chromosomes 13 and 21. Other locations that provide good hybridization targets using probes for repetitive sequences are the distal end of chromosome 1p (Buroker *et al.*, 1987) and the long arm of the Y chromosome (Lau & Schonberg, 1984).

Locus-specific probes identify unique sequences of individual genes or genomic loci. These probes are available in vectors of plasmid, phage, cosmid (Feiss

et al., 1982), P1 (Sternberg, 1990), PAC (P1 derived artificial chromosome) (Ioannou *et al.*, 1994), BAC (bacterial artificial chromosome) (Shizuya *et al.*, 1992), or YAC (yeast artificial chromosome) clones (Burke *et al.*, 1987). These host vectors allow inserts of different sizes to be conveniently incorporated, from a few kb of plasmid or phage clones, to 35-40 kb of cosmid clones, to 80-100 kb of P1 clones, to 100-200 kb of PAC clones, to 100-300 kb of BAC clones, and up to 300-2000 kb of YAC clones. These large-sized locus-specific probes have greatly facilitated the physical mapping of the genome. YAC contig maps covering the whole human genome have been constructed. However, YAC probes are relatively unstable, having low cloning efficiencies and a high degree of chimerism (Cohen *et al.*, 1993). A transformation associated recombination method that utilizes *Alu* and *LINE* repetitive elements of the human genome has been used in the production of YAC probes to lower the level of chimerism (Larionov *et al.*, 1996). P1, PAC and BAC probes also provide more stable alternatives to YAC probes. In addition to physical mapping, the locus-specific probes have been widely used in molecular genetic studies (discussed below), as well as for constructing bar code systems (Uhrig *et al.*, 1999) and matrix-CGH systems (Solinas-Toldo *et al.*, 1997).

Targets

The relaxation level of the target DNA affects the detection resolution. DNA is mostly tightly packed at metaphase and can be resolved to 1-2 Mb (Pinkel, 1999). Mechanically stretched chromosomes (MSCs) have a better resolution of approximately 200 kb (Laan *et al.*, 1995). However, the resolution of MSCs is not uniform across different chromosomes and chromosome regions (Laan *et al.*, 1995). Different methods have been used to produce free DNA fibers on microscopic slides as targets and they are termed as fiber-FISH techniques (Florijn *et al.*, 1995). The resolution using fiber-FISH is around 1 kb (Heiskanen *et al.*, 1996).

Another important use of FISH is to obtain information from interphase nuclei where metaphase spreads are difficult to obtain. Interphase FISH has a resolution level of 50 kb-1 Mb (Trask *et al.*, 1989).

Studies using FISH

Centromere specific probes are often used to detect the chromosome copy number in interphase nuclei (Cremer *et al.*, 1986) or in prenatal diagnosis where numerical

aberrations are the most common abnormalities (Klinger *et al.*, 1992). FISH technique has been used to study the interphase chromosome topography (Emmerich *et al.*, 1989), the spatial relationship between different satellite regions within a chromosome (Rocchi *et al.*, 1991), different models of chromosome aberrations (Lucas & Sachs, 1993), the DNA replication (Rosenberg *et al.*, 1995), numerical aberrations and translocations in metaphase and interphase nuclei (Tkachuk *et al.*, 1990; Losada *et al.*, 1991; Ried *et al.*, 1992), the non-disjunction in sperm (Williams *et al.*, 1993), and the aneusomy in sperm (Spriggs *et al.*, 1995; Martin *et al.*, 1996; Spriggs *et al.*, 1996). The FISH technique has also been applied to determine the location of YAC and cosmid clones by using metaphase, prophase and interphase chromosomes (Lichter *et al.*, 1990; Inazawa *et al.*, 1994; Windle *et al.*, 1995). The location and structure of many genes have been studied using FISH on MSCs and DNA fibers (Vrolijk *et al.*, 1996; Wang *et al.*, 1996).

5.3.3 Other FISH-based techniques

CGH

To cope with the shortcomings of the banding analysis, the CGH technique was invented in 1992 (Kallioniemi *et al.*, 1992; du Manoir *et al.*, 1993). CGH is used to measure the relative abundance of tumor DNA along each chromosome. DNA copy number changes of 10-20 Mb in size can be detected accurately using CGH (Kallioniemi *et al.*, 1994). Amplifications of smaller regions can be detected if they are at least 1 Mb in size and are more than 5-10 fold amplified. Loss of a region 10-12 Mb in size is also detectable if the loss is present in a large portion of the cell population (Bentz *et al.*, 1998). CGH has been successfully utilized in both research and diagnosis [reviewed in (Knuutila *et al.*, 1998; Knuutila *et al.*, 1999)].

The main advantage of the CGH technique is that one does not need to culture the tumor cells. It helps to avoid the potential genetic alterations developed during long-term culturing and the problems associated with the low metaphase yield of malignant cells. CGH can also reveal the genetic composition of marker chromosomes, homogeneous staining regions and double minutes. The disadvantage of CGH is that balanced translocations, inversions, small deletions and polyploid karyotypes are not detectable. In addition, a DNA copy number change has to be present in at least 50% of the cells in the sample for it to be detected (Kallioniemi *et*

al., 1994). This may cause problems in heterogeneous tumors and tumors with normal cell infiltration.

Multicolor fluorescence *in situ* hybridization

To increase the sensitivity of the chromosome banding analysis, several multicolor fluorescence *in situ* hybridization systems were developed in the mid-90s to identify all 24 human chromosomes by different colors. The multiplex-fluorescence *in situ* hybridization (M-FISH) system (Speicher *et al.*, 1996), the spectral karyotyping (SKY) system (Schröck *et al.*, 1996) and the COmbined Binary RAtio (COBRA) labeling system (Tanke *et al.*, 1999) utilize different labeling and detection methods to visualize each chromosome in its own color. The multicolor analysis has been successfully applied to elucidate complex chromosome rearrangements (Veldman *et al.*, 1997). The resolution limit of these systems has not been well established. The current consensus is that aberrations smaller than 2.6 Mb in size are difficult to be detected (Haddad *et al.*, 1998; Uhrig *et al.*, 1999; Azofeifa *et al.*, 2000).

In addition to whole chromosome paints, multicolor analysis using locus-specific DNA probes can create artificially banded chromosomes (bar code) (Lengauer *et al.*, 1993; Chudoba *et al.*, 1999). The bar code system is able to increase the detection sensitivity and accuracy, yield information on mosaicism, and reveal structural rearrangements undetectable by other methods. The combined use of chromosome banding analysis, CGH, and multicolor fluorescence *in situ* hybridization together with the bar code system will give us the maximum cytogenetic information (Uhrig *et al.*, 1999).

5.3.4 Array technology

The array technology is a variation of *in situ* hybridization where hundreds or thousands of targets are attached to a small solid support. The solid support can be either filter or glass (DNA chip). The technology was developed in the late 1990s to make full use of the huge number of gene specific sequences identified by the Human Genome Project, and the abundant information on disease-related gene mutations. In the past few years, the array technology has quickly evolved into a highly technical field involving miniaturization, automation, multicolor fluorescent labeling, and database driven sample and data management (Wilgenbus & Lichter, 1999).

Oligonucleotide array and cDNA array

In an oligonucleotide array, oligonucleotides are either synthesized directly on the surface of the chip, or pre-synthesized and then deposited onto the chip. Several *in situ* synthesis methods have been developed for this purpose and usually synthesize oligonucleotides of less than 25 bases long [reviewed by (Watson *et al.*, 1998)]. In the printing method, pre-synthesized oligonucleotides are covalently attached to chemically treated glasses (Chu *et al.*, 1983; Guo *et al.*, 1994; Joos *et al.*, 1997). The oligonucleotide array is suitable for comparing complementary targets with single base mismatched sequences, such as in the analysis of mutations or single-nucleotide polymorphisms and in mini-sequencing analysis. To study gene expression, multiple oligonucleotides for one gene are needed.

The cDNA array technique is used to study the expression levels of a gene population. cDNA clone inserts are amplified by polymerase chain reaction (PCR) and attached to the solid support utilizing non-covalent charge interactions [reviewed by (Watson *et al.*, 1998)]. The non-covalent nature of the interaction between the target and the solid support may result in the loss of the target clones during the hybridization reaction, leading to reduced sensitivity and inconsistency. However, because the cDNA array is relatively easy to prepare, it is widely in use nowadays.

A variety of applications have been developed using oligonucleotide array and cDNA array, such as the detection of disease-relevant genes and mutations (Hacia *et al.*, 1996; Su *et al.*, 2000), and the characterization of single-nucleotide polymorphisms for linkage analysis (Cargill *et al.*, 1999; Halushka *et al.*, 1999). The technology has also been widely used in gene expression profiling of different physiological status (Ross *et al.*, 2000), linking gene expression patterns to clinical information and cytogenetic abnormalities (Martin *et al.*, 2000; Virtaneva *et al.*, 2000), disease class discovery and prediction (Golub *et al.*, 1999; Alizadeh *et al.*, 2000; Bittner *et al.*, 2000; Kihara *et al.*, 2001), and profiling of the pharmacological and toxicological properties (Nuwaysir *et al.*, 1999; Scherf *et al.*, 2000).

Matrix-CGH

To overcome the limitation of the resolution in conventional CGH, the matrix-based CGH was developed, where hundreds or even thousands of genomic DNA target clones are immobilized on a solid support. The immobilized targets can be cosmid,

P1, PAC, BAC or YAC clones. Matrix CGH has significantly better resolution than conventional CGH. High copy number amplifications can be detected at a resolution of 10 kb and the smallest detectable low copy number gains or losses are approximately 100 kb in size (Solinas-Toldo *et al.*, 1997; Wilgenbus & Lichter, 1999). One can also hybridize the genomic DNA directly onto cDNA arrays, which increases sensitivity to a single gene level (Heiskanen *et al.*, 2000). The matrix CGH technique has been used in gene hunting projects (Albertson *et al.*, 2000) and the detection of disease-relevant genomic imbalances (Bruder *et al.*, 2001).

Tissue array

Another new development in the array field is the tissue array technology. Kononen et al. (1998) described a method to attach up to 1,000 tissue sections onto a slide. The tissue array can be used to study genomic DNA deletions and amplifications (Bubendorf *et al.*, 1999), mRNA expression (Kononen *et al.*, 1998) and tissue immunohistochemistry (Sallinen *et al.*, 2000). The tissue array technique is often used in combination with the oligonucleotide or cDNA array technique. Genes identified in the expression profiling analysis using the oligonucleotide or cDNA microarray techniques can be verified on a tissue array composed of a large number of tumor samples (Moch *et al.*, 1999).

Data management

An essential pre-requisite for the optimal use of microarray data is the development of sufficient tools for collecting, storing, retrieving and querying data, regardless of the technology platform used to generate it (Ermolaeva *et al.*, 1998). The Microarray Gene Expression Database (MGED) group (http://www.mged.org) aims to promote the adoption of standards in array experiments and data. The group has developed recommendations for microarray data annotations. A number of different statistical methods have been investigated for array data analysis. To date, hierarchical clustering analysis has been the most commonly used algorithm applied to array analysis data (Eisen *et al.*, 1998). Other analysis algorithms that have been applied include k-means clustering, Bayesian clustering (Golub *et al.*, 1999), self-organizing maps (Tamayo *et al.*, 1999) and principal component analysis (Hilsenbeck *et al.*, 1999).

6 AIMS OF THE STUDY

- To characterize the deletion in chromosome bands 11q22-q23
  - narrowing down the minimal common region of deletion
  - examining the occurrence of the deletion in different lymphoma subtypes
  - testing of one candidate gene in the region
- To study the gene expression profiles of mantle cell lymphoma and its blastoid variant

7 MATERIAL AND METHODS

7.1 Patients

Altogether samples from 158 lymphoma or leukemia patients were included in this thesis study. Thirty-six CLL samples came from patients attending the CLL out-patient clinic at Tampere University Hospital, and the rest of the samples came from Helsinki University Central Hospital. There were altogether 53 MCL samples, 68 CLL/SLL (small lymphocytic lymphoma) samples, 17 DLBCL (diffuse large B-cell lymphoma) samples, 9 FL (follicular lymphoma) samples and 11 HL (Hodgkin lymphoma) samples. Some of the MCL and CLL/SLL samples were used in more than one study. The patient information is summarized in Table 3. One pathologist classified all the lymphoma samples according to the REAL classification (Harris *et al.*, 1994). The diagnosis and staging of CLL were based on standard clinical, morphological and immunophenotyping criteria (Rai *et al.*, 1975; Binet *et al.*, 1981; Bennett *et al.*, 1989; BCSH, 1994). The histology of the samples and methods used in each study are summarized in Table 4.

Table 3. Patient information

Disease type	No. cases	Age (average)	Sex (F/M)*
mantle cell lymphoma	53	45-90 (65)	20/33
chronic lymphocytic leukemia/ small lymphocytic lymphoma	68	38-84 (64)	20/48
diffuse large B-cell lymphoma	17	40-83 (65)	9/8
follicular lymphoma	9	41-75 (59)	6/3
Hodgkin lymphoma	11	10-76 (37)	7/4

*F, female; M, male.

In study I, paraffin embedded samples from 41 MCL patients were studied by FISH analysis. The samples consisted of 34 primary lymphomas and eight recurrent ones. In one case, both the primary tumor and a recurrent tumor were examined. The samples were from 24 men and 17 women. The patient age at the time of the biopsy ranged from 45 to 87 years (median, 63 years). Four patients had stage I lymphoma at diagnosis, five stage II, one stage III, and 31 stage IV, according to the Ann Arbor classification (Carbone *et al.*, 1971). Seven of the lymphomas were regarded as blastoid variants and two samples contained a mixture of blastoid morphology cells

and typical morphology cells. Nuclei extracted from paraffin-embedded tissue of reactive lymphatic tissues from two individuals were used as the control.

In study II, heparinized peripheral blood specimens were collected from 30 CLL patients and studied by FISH analysis. Twenty-two of the patients were men and eight were women. The age of the patients ranged from 48 to 79 years (median, 64 years). The CLL-scores (Matutes *et al.*, 1994) ranged from 3 to 5. Peripheral blood from two healthy persons was used as the control.

In study III, FISH analysis was performed on 161 samples, comprising 47 MCL samples, 62 CLL/SLL samples, 17 DLBCL samples, 9 FL samples, 11 HL samples and 15 reactive or normal lymph node samples (used as controls). Forty-one MCL and 29 CLL/SLL samples were embedded in paraffin and all the others were either peripheral blood or fresh tissue samples.

In study IV, 36 CLL samples and 37 MCL samples were included. Mutation analysis of the *PPP2R1B* gene was carried out on these samples. DNA and RNA extracted from the peripheral blood of two healthy individuals were used as controls.

In study V, samples from 18 MCL patients were investigated by cDNA array analysis. The age of the patients at diagnosis ranged from 51 to 87 years (median, 65 years). There were 11 men and seven women. Fourteen of the patients (78%) were at Ann Arbor stage III-IV (Carbone *et al.*, 1971) at diagnosis. All the samples used in this study were taken at diagnosis before any treatment. Nine lymphomas were classified as common MCL and nine as the blastoid variant. Total RNA was extracted from deep-frozen tumor tissue specimens. B-cells were purified from adenoid palatine tonsil samples from six healthy children, using microbeads conjugated to monoclonal CD 19 antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany). The pooled total RNA was extracted and used as the control.

Table 4. Histology of the samples and the methods used in studies I – V.

	Disease type	Total case no.	Immuno-phenotypes	No. of positive cases / no. of cases studied	Methods
Study I	MCL	41	CD5+ CD19+ CD20+ CD23- IgM+ IgD+ κ+ λ+ cyclin D1+	38/40 27/27 39/39 28/28 31/31 29/32 12/33 21/33 39/41	FISH
Study II	CLL	30	CD5+ CD22- CD23+ FMC7- undetectable or weak lgκ/λ	26/29 28/29 28/29 29/29 16/29	G-banding analysis, FISH
Study III	MCL	47	CD5+ CD20+ CD23- cyclin D1+	44/46 45/45 30/30 43/47	G-banding analysis, FISH
	CLL/SLL	62	CD5+ CD20+ CD23+	54/57 27/27 52/53	
	DLBCL	17	CD20+	14/14	
	FL	9	CD20+ Bcl-2+	6/6 6/7	
	Classic Hodgkin's lymphoma	7	CD30+ CD15+ CD3+	7/7 7/7 7/7	

	Lymphocyte predominant Hodgkin's lymphoma	4	CD20+ CD15- CD30- CD3+ CD20+ (small cells in nodules)	4/4 3/4 3/4 4/4 2/4	
Study IV	CLL	36	CD5+ CD22- CD23+ FMC7- undetectable or weak Igκ/λ	31/35 34/35 34/35 35/35 19/35	RT-PCR, Sequencing, Single Strand Conformation Polymorphism (SSCP), Southern blotting, Polyacrylamide gel electrophoresis (PAGE)
	MCL	37	CD5+ CD19+ CD20+ CD23- IgM+ IgD+ κ+ λ+ cyclin D1+	34/36 27/27 35/35 25/25 30/30 28/31 12/32 20/32 35/37	
Study V	MCL	18	CD5+ CD19+ CD20+ CD23- IgM+ IgD+ κ+ λ+ cyclin D1+	16/17 15/15 16/16 9/9 17/17 15/18 3/11 14/17 16/18	cDNA array analysis, Real-time PCR

7.2 Immunohistochemistry (study I-V)

The immunohistochemistry was performed on paraffin and / or fresh sections using either the biotin-avidin method or the streptavidin-biotin method. The work was done by the pathologist's laboratory at the time of diagnosis.

7.3 G-banding analysis (study II and III)

G-banding analysis of the samples in study II and III was performed at the time of diagnosis in the Chromosome and Molecular Cytogenetic Laboratory at the Helsinki University Central Hospital. The cells were detached mechanically from fresh lymph biopsies in 5 ml RPMI 1640 medium (Gibco) supplemented with 20% calf fetal serum, 1% glutamine, and 1% penicillin-streptomycin. The cell suspension was treated with colcemid (0.1 µg/ml) in 5% CO_2 at +37˚C for 4 to 12 hours. After hypotonic treatment with 75 mM KCl, the cells were fixed three times in methanol-acetic acid (3:1). Heparinized peripheral blood from leukemia patients was collected and mononuclear cells were isolated and cultured with optimal mitogenic stimulation. After harvesting, the cells were treated hypotonically (0.075 M KCl, 15 minutes, +37˚C) and fixed with methanol-acetic acid (3:1). The cell suspension was spread on slides and air-dried for a couple of days. G-banding analysis was performed according to standard protocols. Images of the metaphase spreads were generated by IKAROS programme (MetaSystems GmbH, Altlussheim, Germany) and ten metaphase spreads of each sample were analyzed. Karyotypes were determined according to the International System for Human Cytogenetic Nomenclature 1995 (Mitelman, 1995).

7.4 Interphase fluorescence *in situ* hybridization (studies I – III)

7.4.1 Sample preparation

Paraffin embedded samples We extracted the nuclei from paraffin embedded tissues according to protocols described by others (Heiden *et al.*, 1991; Hyytinen *et al.*, 1994) with slight modifications. The sections were deparaffinized with xylene 4 times at +55°C and once at +67°C, which was followed by treatment in 100%, 95%, 70% and 50% ethanol series and in H_2O at room temperature. After deparaffinization, sections were digested in 1 ml of Carlsberg's solution (0.1% Sigma protease XXIV, 0.1 M Tris, 0.07 M NaCl, pH 7.2) for 1 hour at +37°C and vigorously shaken in a vortex machine for 20 minutes. The nuclear suspension was filtered through a nylon net (pore size 55 µm) and spread on slides. The nuclei preparations were deparaffinized

again just before the reaction, to ensure high quality hybridization, by incubating the slides at +65°C for 15 minutes and washing them 3 times in xylene at room temperature, followed by dehydration in 100%, 85%, 70% and 50% ethanol series.

Fresh tissue and peripheral blood samples Cells from fresh tumor tissues were separated and cultured overnight in RPMI media [80%RPMI 1640, 20% fetal calf serum, 0.29 mg/ml L-glutamine, 100 mg/ml streptomycin, 100 U/ml penicillin, 0,1 µg/ml Colcemid (all from Gibco, Grand Island, NY, USA)]. Heparinized peripheral blood of leukemia patients was collected and mononuclear cells were isolated and cultured with optimal mitogenic stimulation as described elsewhere (Larramendy *et al.*, 1998). After harvesting, cells were treated hypotonically (0.075 M KCl, 15 minutes, 37˚C) and fixed with methanol-acetic acid (3:1). The cell suspension was then spreaded on slides.

7.4.2 Probes

In study I, 20 YAC probes covering the region 11q22.1-q23.3 contiguously (Figure 1. on page 18) were obtained from the Centre d'Etude Polymorphisme d'Humain (CEPH, Paris, France). All probes were screened with the corresponding markers using PCR and FISH analysis to confirm their identities and locations. None of the probes showed chimerism, as all hybridized only to 11q22-q23. YAC probes 808c6 and 953e4, hybridizing to 11p13, were used as controls of hybridization efficiency and for evaluating the chromosome copy number. Seventeen MCL cases were tested with all 20 probes. Twenty-four MCL cases were screened with probe 755b11 first. Cases found to have the 11q23 deletion were tested with a wider set of probes from the YAC contig to determine the extent of the deletion. Probe 981g12, which represents the region containing the *ATM* gene, was tested on cases that did not show the deletion of the region represented by YAC755b11 to eliminate the possibility that the deletion in 11q23 was not uniformly detected by YAC755b11.

In study II, based on the results from study I, eight YAC clones (793d9, 755b11, 913g9, 957e4, 771d4, 939b12, 785e12, and 911f2) were used. These YAC probes constitute a contig of 7.8 Mb in size spanning the region 11q23.1-q23.3 (Figure 1. on page 18). All of the YAC probes hybridized only to 11q22-q23 and none of them showed chimerism. YAC 953a4 (CEPH-Généthon), mapped to 11p13, was

used as the chromosome copy number control. The identities of the YAC probes were confirmed by PCR with their corresponding markers.

In study III, YAC probe 755b11 was used.

The yeast cells containing the probes were grown in AHC medium for 4-6 days and DNA was extracted from the yeast by using a glass bead and phenol based procedure (Hoffman & Winston, 1987).

7.4.3 Dual-color and single-color FISH

In dual color FISH, the control and test DNA were labeled with digoxigenin-dUTP and biotin-14-dATP, respectively, by nick-translation according to the standard procedure. In single color FISH, the DNA was labeled with biotin-14-dATP. The probe mixture contained approximately 1 µg labeled DNA, 25 µg human Cot-1 DNA (Gibco) and 25 µg herring sperm DNA (Sigma Chemical Co., Bornem, Belgium) dissolved in 50% formamide and 10% dextran sulfate in 2×SSC. The probe mixture was denatured at +75˚C for 5 minutes before the hybridization.

The nuclei preparations extracted from paraffin embedded samples were first deparaffinized as described on page 35-36. In order to increase the hybridization efficiency, the slides were treated in 1 M sodium thiocyanate at +70°C for 15 minutes, followed by treatment in 0.05 N HCl at +37°C for 10 minutes and in 5 mg / ml pepsin dissolved in 0.05 N HCl at +37°C for 20 minutes. The slides were then denatured in 70% formamide / 2 × SSC (pH 7) at +75°C for 5 minutes. The fresh tissue or blood samples preparations were treated with 0.01 N HCl at +37°C for 5 minutes and with 5 mg / ml pepsin dissolved in 0.01 N HCl at +37°C for 7 minutes, and then denatured in 70% formamide / 2 × SSC (pH 7) at +65°C for 2 minutes. The denatured probe mixture was hybridized onto the slides and the reaction continued for at least two days in a moist chamber at +37°C.

After the hybridization reaction, the slides were rinsed once in 2 × SSC and twice in 0.1 × SSC at +45°C for 5 minutes each. In dual-color FISH, the biotinylated test DNA was detected by incubating the slides at +37°C with the avidin-TRITC solution (1:5000, Vector laboratories, Burlingame, CA, USA), the biotinylated anti-avidin solution (1:1000, Vector) and the avidin-TRITC solution (1:5000) for 40, 45, and 40 minutes, respectively. Simultaneously, the digoxigenin-labeled control DNA was visualized with the mouse-anti-digoxigenin solution (1:100, Boehringer

Mannheim, Germany), the rabbit-anti-mouse FITC solution (1:300, Sigma, St. Louis, MO, USA) and the goat-anti-rabbit FITC solution (1:200, Sigma) for 60, 40, and 40 minutes, respectively. In single-color FISH, the biotinylated DNA was detected by incubation at +37°C with the avidin-FITC solution (1:200, Vector), the biotinylated anti-avidin solution (1:1000, Vector) and the avidin-FITC solution (1:200) for 40, 45, and 40 minutes, respectively. The preparations were counter-stained in 2 μg/ml of DAPI for 10 minutes.

From each preparation a minimum of 200 morphologically intact and non-overlapping nuclei were checked using a Leitz fluorescence microscope (Laborlux D, Germany). The locus was considered deleted if the frequency of cells with only one signal was higher than the cut-off value. The cut-off value for deletion was obtained by experiments on normal reactive lymphoid tissue samples. The cut-off level was defined as the mean value of the frequencies of nuclei exhibiting one signal plus three times the standard deviation.

7.5 RNA and DNA extraction (study IV and V)

Total RNA of mononuclear cells collected from heparinized blood of CLL patients was isolated using the TRIzol Reagent (Life Technologies Inc., Grand Island, NY, USA) according to the manufacturer's instruction (study IV). Total RNA was isolated from lymphoma specimens preserved at –120 ˚C using the RNeasy®Mini Kit from QIAGEN (Valencia, CA, USA) according to the manufacturer's instruction (study V). For array analysis, the total RNA was treated with DNase according to the manufacturer's instruction (Clontech Laboratories Inc., Palo Alto, CA, USA). The purity and the integrity of the RNA were examined by electrophoresis on 1% agarose gel. Only high quality RNA was used for gene expression analysis.

DNA was extracted from deep-frozen tissue, blood or bone marrow samples from CLL and MCL patients, using the phenol-chloroform extraction method (study IV). In four cases of MCL, DNA was extracted from archival paraffin-embedded material according to the protocol described elsewhere (Isola *et al.*, 1994).

The RNA and DNA of the control samples were extracted using the same method as the patient samples in each study.

7.6 RT-PCR analysis (study IV)

We used RT-PCR to detect mutations in the *PPP2R1B* gene using total RNA from ten CLL patient samples in study IV. 0.8 µg RNA was reverse-transcribed to cDNA in a 20 µl reaction mixture using the standard random priming method with M-MLV reverse transcriptase (Promega, Madison, WI, USA) and RNAse inhibitor (Promega) according to the manufacturer's instruction. The subsequent PCR analysis was usually performed using 3 µl cDNA, 1 × PCR buffer (Perkin-Elmer, Branchburg, NJ, USA), 250 µM dNTPs (Finnzymes, Espoo, Finland), 0.8 µM forward and reverse primers, and 2 units of AmpliTaqGOLD polymerase (Perkin-Elmer) in a final volume of 50 µl. The $MgCl_2$ concentration was 1.5 mM. Primers were designed using the primer 3 program (http://www-genome.wi.mit.edu/).

7.7 PCR-SSCP analysis (study IV)

PCR-SSCP is a method to detect mutations, polymorphisms and sequence variants, utilizing the property that the electrophoretic mobility of single-stranded nucleic acid depends both on the length and the sequence of the target. We used PCR-SSCP to detect genomic mutations in the *PPP2R1B* gene in study IV. Primers for PCR analysis on genomic DNA were designed using the primer 3 program (http://www-genome.wi.mit.edu/). The reactions were carried out in a 50 µl reaction volume containing 100 ng of genomic DNA, 1 × PCR buffer (Perkin Elmer), 250 µM dNTPs (Finnzymes), 0.6 µM forward and reverse primers, and 2 units of AmpliTaqGOLD polymerase (Perkin Elmer). The concentration of $MgCl_2$ was optimized to 1-2.5 mM in the reaction mixture for each exon and 6% DMSO was used in reactions for exon 1. In reactions for exon 8, the concentrations of the forward and reverse primers were 0.5 µM and 1µM, respectively.

After PCR, equal volumes of the reaction mixture and the denaturing loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue and 0.05% xylene cyanole) were mixed. The mixture was denatured for 5 minutes at +95°C and loaded onto a 0.4 mm × 30 cm × 45 cm gel. The gel contained 0.5 × mutation detection enhancement gel solution (FMC Bioproduct, Rockland, ME, USA) and 1 × TBE buffer. The gels were electrophoresized at 3-6 W overnight and then stained with silver according to the standard protocol.

7.8 Sequencing PCR products (study IV)

In order to determine the nucleic acid sequences in mutated samples, we sequenced PCR products showing aberrant bands in either the RT-PCR analysis or the PCR-SSCP analysis in study IV. The PCR product was purified using a QIAquick PCR purification kit (Qiagen) and DNA fragments were sequenced using an ABI PRISM Dye Terminator (Perkin Elmer) according to the manufacturer's instruction. Cycle sequencing products were then electrophoresized on 6% Long Ranger gels (FMC Bioproduct) and identified using an Applied Biosystems model 373A automated DNA sequencer (Perkin Elmer). The DNA fragments were sequenced using both forward and reverse primers.

7.9 Southern blotting analysis (study IV)

In some samples PCR-SSCP analysis was not successful in consecutive exons. In order to see if this was due to deletions spanning more than one exon in the samples, we performed the Southern blotting analysis. EcoR I (New England Biolabs, Beverly, MA, USA) and TaqI (New England Biolabs) restriction enzymes were used to digest genomic DNA (10 μg). The digested DNA was fractionated according to size in 0.8% agarose gel (Bio-Rad, Hercules, CA, USA). After denaturation and neutralization treatment, DNA fragments were blotted onto Hybond-C extra filters (Amersham, Piscataway, NJ, USA). A cDNA probe containing *PPP2R1B* exons 1-9 was labeled radioactively with [α^{32}P] dCTP using standard methods and hybridized to the DNA on the filters. After hybridization, the filters were washed at least twice in 3 × SSC and 0.1% SDS at +65°C for 20 minutes each.

7.10 PAGE analysis (study IV)

After PCR-SSCP analysis, we found abnormal bands in exon 1 of the *PPP2R1B* gene in some samples. To confirm this finding, we performed the PAGE analysis. PAGE was performed using Ultrapure Sequagel (National Diagnostics, Atlanta, GA, USA). The gels were silver-stained. The reactions were performed according to the standard protocol.

7.11 cDNA array analysis (study V)

In order to study the gene expression profiles of common and blastoid variant MCL, we performed cDNA array analyses using the Atlas Human Hematology/Immunology

cDNA expression array (7737-1) (Clontech Laboratories Inc). The array includes 406 human genes and nine housekeeping genes immobilized in duplicate dots on a nylon membrane. The 406 genes included were previously shown to be involved in various hematological and immunological disorders or in the normal functions of the human immune system. The names of the genes and their coordinates on the filter are available on-line (http://www.clontech.com/atlas/genelists/index.shtml).

Total RNA (3 - 4 μg) was reverse-transcribed into cDNA with primers provided with the reaction kit by Clontech Laboratories Inc. and was labeled with ^{33}P-dATP. Probe purification and hybridization to the array were performed according to the manufacturer's instruction. After the post-hybridization washes, the array was exposed to an imaging plate (BAS-MP 2040S; Fuji, Tokyo, Japan) at room temperature for 3-5 days and scanned by a phosphorimager (Bio-Imaging Analyzer, BAS-2500; Fuji). The image was analyzed and the intensity of each spot was quantified using the Atlas Image 1.5 software (Clontech Laboratories Inc.). The background was determined as the average intensity of the blank space between the different panels of the array. Saturation of the hybridization signal was not observed. All filters used were from the same batch to minimize printing variation between batches.

7.12 Gene expression data analysis (study V)

Three statistical analysis methods were used to analyze the array data: the regression analysis, the principal component analysis (PCA) and the naive Bayes classifier. The first two methods focused on identifying deregulated genes and the third one provided a model for the differential diagnosis of common MCL and its blastoid variant.

In regression analysis, Microsoft Excel 2000 software was used to generate the xy-scatterplot of expression data from array experiments on one sample and the control. The same software was used to determine the least-squared regression line and the Pearson correlation coefficient of the scatterplot. The 95% confidence interval around the regression line was determined. Spots beyond this area were identified as abnormally expressed (Smid-Koopman *et al.*, 2000). If a gene was found abnormally expressed in more than 3 samples, it was considered deregulated in MCL. By comparing the frequencies at which a gene was identified as abnormally expressed in

common and blastoid variant MCL samples, we were able to identify genes that were differentially deregulated in the two types of MCL.

The Principal Component Analysis (PCA) method combined the expression data from all 18 samples and compared it to the control. Each gene was given a score. Genes with a score around zero were considered normal and genes with big positive or negative scores were considered deregulated. Technically, from the absolute intensity of each gene, the background intensity was first subtracted, followed by the subtraction of the control intensity, then the mean value of the data set of an array was subtracted from each data point. To give equal weighting to all cases, the data in each case was standardized to unit variance by dividing all the values by the standard deviation. The covariance matrix is estimated from the data and the principal components are given by eigenvectors and associated eigenvalues of the covariance matrix. The first principal component is the eigenvector that has the largest eigenvalue of the covariance matrix. The first principal component is interpreted as the average expression level of each gene (Hilsenbeck *et al.*, 1999). The gene were scored based on their first principal component projections.

The naive Bayes classifier has been widely used in diagnostic applications (Duda *et al.*, 2001). In this method, we assume that the expression level of each differentially deregulated gene in each class is distributed according to a normal distribution. We may estimate the parameters of the normal distribution. The parameters for each class distribution are the mean vector and the diagonal covariance matrix. The parameters were estimated by the method of maximum likelihood by assigning the average value and the diagonal covariance matrix of the sample to the mean vector and the covariance matrix of the class densities, respectively. Once the parameters of the classifier have been estimated, the model can be used for classification. In order to assess how well the classifier would operate in a clinical setting, where cases with unknown diagnosis are encountered, we applied the leave-one-out cross-validation method (Golub *et al.*, 1999). In this case, we calibrated a new classifier based on data from 17 samples and assessed its accuracy with the 18th sample, and repeated this effort 18 times with all possible combinations to assess the accuracy of the classifier. The cross-validated test error is the average of all errors made during the testing phase. To check whether the classification results are significant, we performed a randomization experiment. The class labels of the

observations were randomly permuted, and a new classifier was derived using the same method as for the original data. The accuracy of the new classifier is compared to the original classifier. This procedure was repeated 10.000 times.

7.13 Real-time PCR analysis (study V)

Real-time PCR utilizes fluorescence labels to detect PCR products. Thus, the reaction process (in terms of PCR product amount) can be followed at real time and the product can be quantified. In study V, we used real-time PCR to confirm the array results. Four genes were tested, namely *AF17*, *ADA*, *RGS2* and *CMYC*, by the real-time PCR analysis. 820 ng purified RNA was reverse-transcribed using the 1st strand cDNA synthesis kit for RT-PCR (AMV) (Roche Diagnostics Corp., Indianapolis, IN, USA). The cDNA was diluted 10-fold prior to PCR amplification. Real-time PCR was performed using the LightCycler rapid thermal cycler system (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instruction. The primers were designed by TIB Molbiol (Berlin, Germany).

Reactions were performed in a 10 µl volume with primer concentrations of 0.5 µM each, Mg^{2+} concentration optimized between 2-5 mM, and 1 µl of cDNA. The LightCycler-FastStart DNA Master SYBR Green I kit was used (Roche Diagnostics GmbH, Mannheim, Germany). To confirm amplification specificity, the PCR products from each primer pair were subjected to the melting curve analysis and subsequently gel electrophoresis analysis.

8 RESULTS

In this thesis study, we first tried to identify the critical regions in 11q22-q23 using FISH analysis on samples of MCL and CLL. We identified two critical regions, one of which was the minimal common region of deletion in 11q22-q23. We then used the probe representing this minimal common region of deletion to study the occurrence of the deletion in 11q22-q23 in different lymphoma subtypes. In study IV, we performed mutation analysis on one candidate gene in 11q23. Finally in study V, we investigated the gene expression profiles of common and blastoid variant MCL using the cDNA array analysis.

8.1 Deletion in chromosome bands 11q22-q23 in mantle cell lymphoma (study I)

We studied 41 MCL samples by FISH analysis in study I. We first screened 17 MCL samples with 20 YAC probes covering the whole area of 11q22.1-q23.3. We found deletions in this area in nine samples (53%). The deletion was usually large, spanning several megabases, although in one sample, only the region represented by YAC755b11 (1.6 Mb) in 11q23.1 was deleted. We then screened additional 24 MCL samples with the probe 755b11 and found deletions in 11 samples, yielding an overall frequency of deletion of 49% in all samples (20/41). The deletion was present in at least 25% of the nuclei examined and in most samples it occurred in more than 60% of the nuclei. In one case, a large biallelic deletion was detected. Two signals were seen with a control probe in more than 78% of the cells.

The region represented by the YAC probes 785e12 and 911f2, at the distal end of 11q22-q23, seemed to be rather critical, because the deletion ended here in most of the informative cases (12/19, 63%). In two cases, the end point of the deletion occurred between the region represented by probes 911f2 and 822g8. In one case, it was between the region represented by probes 939b12 and 785e12.

The proximal end point of the deletion was not determined, because the deletion usually extended beyond 11q22.1. In some lymphoma samples, two or three signals were found by probes 711d4 and 939b12 while the flanking regions were deleted.

We studied the association between the presence of a deletion in this region and clinical parameters. No association was found between the presence of a deletion and median age, gender, International Prognostic Index, or Ann Arbor stage of the patients.

8.2 Discontinuous deletion in 11q23 in chronic lymphocytic leukemia (study II)

No numerical abnormality or translocation involving chromosome 11 was found in any of the CLL cases. Standard cytogenetic analysis revealed deletions in 11q in six samples (nos 1, 2, 9, 11, 18 and 20). Five of these samples had other chromosome abnormalities. FISH analysis confirmed these cytogenetic findings and revealed three more samples (nos 3, 25 and 30) with the deletion in 11q23. The overall frequency of 11q deletion was therefore 30% (9/30). In each of the samples with the deletion in

11q23, the percentage of cells showing deletions ranged from 22% to 93% (mean, 77%). Five samples had discontinuous deletions.

The genomic region represented by the YAC probes 793d9, 755b11 and 913g9 was deleted in all the samples that had the deletion. The region represented by YAC 785e12, located about 4 Mb distal from 755b11, was deleted in all but one of the samples (no. 2). The area in between, represented by YAC probes 957e4, 771d4 and 939b12, was deleted discontinuously in five samples (case nos 3, 9, 18, 20 and 30). In sample nos 3, 9 and 30, the region represented by YAC 785e12 was deleted while both of flanking regions were not.

In sample nos 3 and 9, two signals were seen at their normal positions in 11q when probes 771d4, 939b12 and 911f2 were hybridized to the metaphase spreads of the samples, confirming that there was no deletion of these regions in these samples. The same was true when sample no. 24 was hybridized with probes 771d4 and 939b12. Dual-color FISH on metaphase spreads from sample nos 3, 9, 18 and 30, with probes 755b11 and 785e12, showed that the deletions in these regions affected the same chromosome. No signal splitting was found in sample nos 3, 9 and 30 when dual-color FISH was performed on interphase cells with probes 939b12 and 911f2, suggesting that there were no translocations involving these regions.

8.3 Deletion in 11q23 in different lymphoma subtypes (study III)

We studied 146 lymphoma or leukemia samples and 15 control samples by FISH analysis in study III and found deletion of the region represented by YAC755b11 in 40 samples (25%). MCL had the highest deletion frequency with 23 out of 47 samples (49%) having the deletion. No significant difference in frequencies were found between common and blastoid variant MCL. Out of the 62 CLL/SLL samples studied, 13 (21%) had the deletion. The deletion is more common in the blood specimens of CLL/SLL (9 out of 30, 30%) than the lymph node specimens of CLL/SLL (4 out of 32, 13%). Four out of the 17 (24%) DLBCL samples were found to have the deletion. There were three cases of Richter's syndrome among the CLL/SLL and the DLBCL patients, and they all had the deletion. None of the nine FLs, 11 HLs or the 15 reactive or normal lymph node specimens had the deletion. The percentage of cells showing the deletion ranged from 22% to 95% (mean, 72%).

No significant difference was found in the deletion frequencies between men and women (MCL, p=0.86; CLL/SLL, p=0.74; DLBCL, p=1.00). Neither was there difference in age at diagnosis between the cases with or without the deletion in any of the subtypes of lymphoma (MCL, p=0.89; CLL/SLL, p=0.17; DLBCL, p=0.82).

The deletion was more common in MCL than in other types of lymphoma (MCL vs CLL/SLL: p=0.0021; MCL vs DLBCL: p=0.069). The difference in deletion frequencies between CLL/SLL and DLBCL was not statistically significant (p=1.00).

Although the deletion frequency is higher in blood samples than in lymph node samples of CLL/SLL (30% vs 13%), the difference is not statistically significant (P = 0.045).

8.4 Mutation analysis of the *PPP2R1B* gene (study IV)

Ten CLL samples were subjected to the RT-PCR and sequencing analysis. Exon 3 of the *PPP2R1B* gene was found deleted in sample no. 16.

We performed PCR-SSCP analysis on the genomic DNA of 26 additional CLL samples and 37 MCL samples. Ninety-six percent of the PCR experiments were successful. After the SSCP analysis, no abnormality was found in exons 2-15 in any of the samples. One CLL sample (No. 52) showed abnormal bands in the analysis of exon 1, which was confirmed by the PAGE analysis. The sequencing analysis showed that this abnormality was a silent mutation CTA (Leu)- CTG (Leu) at codon 22.

To see if there were large deletions spanning several exons, we did Southern blotting analysis on 20 MCL samples and 18 CLL samples (two of the CLL samples studied by the RT-PCR analysis, nos 16 and 41, were also included in the RT-PCR analysis). Experiments on DNA digested by TaqI yielded informative results from 8 MCL samples and 17 CLL samples. Experiments on DNA digested by EcoRI yielded informative results from seven MCL samples but failed in all CLL cases because of the poor quality of the DNA. A structural rearrangement of the *PPP2R1B* gene was found in one MCL sample (no. 33). Details of this rearrangement could not be studied because RNA was not available.

Altogether, therefore, we found two abnormalities in the *PPP2R1B* gene, a deletion of exon 3 in one CLL sample (no. 16) and a structural rearrangement in one MCL sample (no. 33).

8.5 The gene expression profiles of MCL and its blastoid variant (study V)

The regression analysis identified 46 deregulated genes. Thirty-four of them were found to be deregulated in both common and blastoid variant MCL at frequencies ranging from 22% to 100%. The remaining 12 genes were found to be differentially deregulated in common and blastoid variant MCL. In PCA, we chose 20 genes with the highest scores and 20 genes with the lowest scores as the deregulated genes for MCL. Genes identified by both the regression analysis and PCA were considered deregulated in both common and blastoid variant MCL. There were 18 such genes (Table 5).

Table 5. Genes deregulated in both common and blastoid variant MCL

Gene name	Chromosome location	Status	Suggested role in MCL
SCYA21	9p13	Up	Normal body function
GPR13	3p21.3	Up	Not known
BCL1	11q13	Up	Oncogene
AF17	17q21	Up	Not known
MIG	4q21	Up	Normal body function
CD5	11q13	Up	Not known
ANX2	15q21-q22	Up	Tumor metastasis promoter
CD44H	11p13	Up	Tumor metastasis promoter
TP12/CD6	11q13	Up	Not known
TCF7	5q31.1	Up	Not known
GRN	17q21.32	Up	Oncogene
GZMK	5q11-q12	Up	Normal body function
EBI2	13	Down	Not known
ADA	20q12-q13.11	Down	Not known
CALLA/CD10	3q25.1-q25.2	Down	Oncogene
CD66D	19q13.2	Down	Tumor suppressor
RGS1	1q31	Down	Tumor metastasis promoter
RGS2	1q31	Down	Tumor metastasis promoter

The expression data of the 12 genes differentially deregulated for common and blastoid variant MCL were used to calibrate the naive Bayes classifier. The leave-one-

out cross-validation tests showed correct classification in 14 out of the 18 cases. Therefore, the cross-validated test error for the classifier was 0.222. The randomization experiments yielded a significance level of 0.028.

To improve the classification, we used subsets of the 12 genes to calibrate the classifier. This had not only the potential to provide a more accurate classifier, but was also a way to choose and confirm the differentially deregulated genes. We tested gene sets consisting of one to eleven genes with different gene composites. It turned out that the best classifier is a gene set consisting of eight genes. The mean classification error was 0.111. Randomization results yielded a significance level of 0.018. Therefore, we conclude that these eight genes are significant in separating the blastoid variant from common MCL and they are differentially deregulated in common and blastoid variant MCL (Table 6).

Table 6. Genes differentially deregulated in common and blastoid variant MCL

Gene Name	Chromosome location	Status	Deregulated in common MCL	Deregulated in MCL blastoid variant	Suggested role in MCL
CMYC	8q24.12-q24.13	Up	0	44%	Oncogene
BCL2	18q21.33	Up	33%	56%	Oncogene
TOP1	20q12-q13.1	Up	67%	11%	Not known
CD45	1q31-q32	Up	56%	0	Tumor suppressor
CD70	19p13	Up	22%	56%	Oncogene
NFATC	18q23	Up	56%	11%	Functions through downstream targets.
PIM1	6p21.2	Up	0	56%	Oncogene
CD23	19p13.3	Down	33%	67%	Not known

To confirm the cDNA array results, we did real-time PCR analysis on four genes, *AF-17*, *ADA*, *RGS2* and *CMYC*. The results were in accordance with the array analysis results.

9 DISCUSSION

9.1 Discussion of the techniques applied

Control samples

We used lymph node biopsy specimens from two healthy individuals as controls in study I, and peripheral blood specimens from two healthy individuals as controls in study II. The control samples were tested by 20 YAC probes in study I and 8 in study II. In each experiment, a total of 200 nuclei were analyzed. The frequency of normal nuclei with one-signal in each experiment was determined. The average frequency and the standard deviation were calculated, and the cut-off level was determined as the average frequency plus three times the standard deviation. If the frequency of nuclei with one-signal in a FISH experiment on one patient sample is higher than the cut-off level, we consider the region represented by the probe is deleted in the sample.

We studied 15 normal or reactive lymph node specimens with YAC755b11 in study III. In addition to determine the cut-off level, these samples were used to determine the occurrence of the deletion in 11q23 in normal lymph node tissues.

In study IV, we used DNA and RNA extracted from the peripheral blood of two healthy individuals as controls. To determine whether a point mutation is a polymorphism, one usually needs a larger series of normal samples. In this study, no point mutation was found. Therefore, we think two control samples are sufficient.

Choosing the proper control is perhaps the most difficult part of the array analysis. In many cancer types, the normal cell counterparts are still unknown or controversial. In addition, to get enough amount of the normal cell counterpart from healthy individuals is often difficult. In study V, we used the B-cells purified with CD19-coupled microbeads from adenoid palatine tonsil samples from six healthy children as the control. Whether this control is the most suitable normal cell counterpart of MCL malignant cells can be argued. Theoretically, the normal cell counterpart of MCL malignant cells is the CD5+ B-cell from the mantle zone of lymph nodes. The B-cells extracted from tonsils are normally CD5-. The CD19 is a pan-B-cell marker, which is expressed not only on mantle zone B-cells, but also on germinal center and marginal zone B-cells. In addition, the patients were mostly over 60 years old, while the control samples were extract from children. However, to get enough CD5+ cells from the mantle zone of lymph nodes from healthy old men is

practically impossible. The alternative could be CD5+ B-cells enriched from the blood. But a recent study by Rosenwald et al. (2001) (Rosenwald *et al.*, 2001) has shown that there is no similarity in gene expression profiles between CD5+ B-cells enriched from blood and CLL malignant cells. CLL is another disease whose normal cell counterpart is proposed to be the CD5+ B-cell. Rosenwald et al. (2001) suggested that the gene expression profile in CLL might be a manifestation of oncogenic properties and therefore might not be a feature of any normal B-cell population. Although the same kind of study has not been conducted in MCL, we think that similar conclusions can be drawn for MCL as well. Based on these facts, we think our choice of the control is suitable for this study. We were, however, careful when interpreting the deregulated genes. We used two statistical analysis methods to confirm the genes identified as deregulated: regression analysis and PCA in identifying genes deregulated in both common and blastoid variant MCL; regression analysis and naive Bayes classifier in identifying genes differentially deregulated in common and blastoid variant MCL. The stringent selection criteria guaranteed that the genes chosen by both methods were indeed deregulated. However, we should be aware that this selection process might have eliminated genes with low degree of abnormality but of biological importance. Therefore, genes chosen by one statistical analysis method as deregulated but not by the other also deserve attention.

Interphase FISH

Either dual-color or single-color interphase FISH was applied in studies I, II and III. One of the biggest advantages of interphase FISH is that we can utilize the paraffin-embedded archival material. Since the paraffin-embedded material is much more abundant than the fresh or deep-frozen one, interphase FISH enables us to conduct the research on larger series of patient material. The disadvantage of interphase FISH is that it is rather labor-intensive, especially when multiple probes are tested on large sample series. Over 700 FISH experiments were performed in this thesis study, which has been both time and money consuming. Therefore, it is necessary to combine interphase FISH with other high-throughput methods, such as the tumor array technology, in order to increase the efficiency.

Mutation analysis

We used PCR-SSCP as the main method for detecting mutations in the *PPP2R1B* gene. PCR-SSCP is a popular electrophoretic method for the detection of mutations. It is technically simple, with high capacity and relatively high sensitivity. There are a number of factors affecting the sensitivity of PCR-SSCP: choice of matrix, electrophoretic conditions, presence of additives, and the fragment size and GC content of the sample. It has been shown that the sensitivity of PCR-SSCP is between 70% and 95% depending strongly on the factors described above (Moore *et al.*, 2000). Except the sample's fragment size and GC content, we optimized all the other factors for each exon before the experiments. Mutation of one nucleotide was detected in our study, proving the sensitivity of our experiments. However, we can not exclude the possibility that some mutations were not detected because PCR-SSCP experiment conditions were not optimal for some exon sequences.

Perhaps the most sensitive method for mutation detection is the sequencing of the cDNA transcribed from RNA. However, the availability of RNA has often been the restricting factor. Direct sequencing of the genomic DNA (including exons and exon-intron boundaries) is also a very sensitive method. However, it is very costly to conduct such analysis on large series of samples, especially when the gene under investigation has many exons. In addition, direct sequencing of genomic DNA cannot detect large deletions spanning more than one exon. Thus, assisting methods, such as Southern blotting analysis, have to be used to make the investigation complete.

Statistical methods used in the array analysis

In most of the array studies, the ratio of a gene's expression level in tumor samples to its expression level in the control sample plays an important role (Eisen *et al.*, 1998). The glass-based array system is very convenient and accurate, because both the tumor RNA and the control RNA are simultaneously hybridized onto the array, leading to minimal inter-experimental variations. The inter-experimental variations are considerably higher using the filter-based array system, because the tumor and control RNA are hybridized to the array in two independent experiments. Therefore we needed analysis methods that could best solve the normalization problem. The regression analysis treats the expression levels of all genes in an array experiment as a population and compares it with another population. The coefficients a and b in the

regression function y = ax + b should, in principle, explain the major inter-experimental variants, namely the overall intensity variation caused by the different amounts of RNA used, and the background variation caused by different exposure time. In PSA, we subtracted the average value of all gene expression data on an array from the expression data of each gene, then divided this figure with the variance. This is also a powerful way to normalize the expression data.

The regression analysis is, however, rather laborious, especially if there are a large number of samples. Another disadvantage was the use of frequencies, which are sensitive to the choice of thresholds. The PCA is less labor intensive. It is also more powerful in treating the sample set as a group.

There are enormous interests in the tumor class prediction and classification field. Most impressively, many creative statistical analysis strategies are being applied. In our study, we used the naive Bayes classifier. This method and its variants have been applied to the classification of cancer before (Golub *et al.*, 1999; Dudoit *et al.*, 2000). We were able to verify the accuracy of this classifier by the cross validation tests. Although it is statistically sufficient, the true value of this classifier will only be shown when new MCL cases are correctly diagnosed. It is conceivable that with more samples and an array containing more genes, we will be able to gain much more information about the disease and generate a more accurate classifier.

9.2 The minimal common region of deletion in 11q22-q23 (study I, II)

By utilizing the YAC-contig, we wanted to narrow down the critical regions in 11q22-q23. In study I, the minimal common region of deletion in 11q22-23 was found to be the region represented by YAC755b11. In study II, the same genomic region was involved in all cases with the deletion. In one case, the region represented by YAC755b11 was deleted more frequently (63%) than the flanking regions (42% and 31%, respectively). This suggested that part of the cell population had the deletion only in the region represented by YAC755b11. Similar results have been obtained by others (Stilgenbauer *et al.*, 1996). The YAC755b11 probe was also used to screen 214 CLL cases. Deletions were found in the cases that had an inferior prognosis (Döhner *et al.*, 1997). These data suggested that 11q23.1 might contain a tumor suppressor gene that could be important in the pathogenesis of both MCL and CLL. Our results support the conclusion that this tumor suppressor gene is located in the region represented by YAC755b11. However, the study by Stilgenbauer et al. (1999) on 81

MCL cases showed that the minimal common region of deletion in 11q23 was the one represented by YAC801e11, a region more proximal than the region represented by YAC755b11 (Stilgenbauer *et al.*, 1999). This is the region where the *ATM* gene is located. Subsequent analysis of the same samples confirmed the inactivation of the *ATM* gene, leading to the conclusion that the *ATM* gene was the proposed tumor suppressor gene in 11q23 (Schaffner *et al.*, 2000). Nevertheless, the significance of the region represented by YAC755b11 should not be neglected. It is highly possible that there is one yet unknown tumor suppressor gene in this region, as was evidenced by our results and the translocations found by Stilgenbauer et al. (1996) in this region. Additional evidences came from the mutation analysis of the *ATM* gene in CLL, where mutation was not found in a fraction of CLL cases that had the monoallelic 11q23 deletion (Schaffner *et al.*, 1999).

9.3 The second critical region in 11q22-q23 (study I, II)

In addition to the region represented by YAC 755b11, we found another critical region in 11q23. In study I, the distal end point of the 11q22-q23 deletion was found to be between the regions represented by YACs 785e12 and 911f2 in the majority of samples studied (63%). Interestingly, the two regions were found deleted in cases where the deletion was discontinuous. In study II, the region represented by YAC785e12 was deleted in eight out of the 30 samples studied (27%), and in three samples this region was deleted while the flanking regions remained intact. Our results suggested that the region represented by YAC785e12 and its vicinity were the second critical region in 11q22-q23. The region might contain another yet unknown tumor suppressor gene.

YACs 785e12 and 911f2 are mapped to 11q23.3, the region that often contains structural aberrations and rearrangements in hematological malignancies (Heim & Mitelman, 1995). This region also contains inherited and constitutionally fragile sites, which makes it susceptible to chromosome breakage and rearrangements (Sutherland *et al.*, 1983; Yunis & Soreng, 1984). In addition to hematological diseases, loss of heterozygosity of the marker APOC-3 that is located in the region represented by 785e12 has been found frequently in breast carcinomas (Laake *et al.*, 1997) and cervical carcinomas (Hampton *et al.*, 1994b) (frequencies of 45% and 43%, respectively), suggesting the existence of a tumor suppressor gene in this region.

In our study, the deletion in 11q23 in one sample did not involve the region represented by YAC 785e12. Similarly, Stilgenbauer et al. (1996) have reported that seven out of 18 CLL samples in their study did not have the deletion of this region. One explanation might be that a part of this region could have been deleted but was beyond the resolution limit of FISH. In study II, dual-color FISH on metaphase spreads with YACs 785e12 and 755b11 showed that the deletion of the two regions occurred in the same chromosome, implying that the deletion of the region represented by YAC785e12 might be a secondary event associated with the deletion of the region represented by YAC755b11. The deletion of the region represented by YAC785e12 might therefore be a late event in the disease progression and might not be present in cases at early stages of the disease.

A tumor suppressor gene, *TSLC1*, was identified recently in non-small-cell-lung cancer. This gene is located in the central 100-kb fragment of the region represented by YAC939b12, which is very close to the region represented by YAC785e12 (Murakami *et al.*, 1998; Kuramochi *et al.*, 2001). It will be interesting to see if this gene has a role in the pathogenesis of MCL and CLL.

9.4 The 11q23 deletion in different lymphoma subtypes (study I, II, III)

Our results have shown that the deletion in 11q23 is not uniformly present in NHL. We studied 47 MCL samples in studies I and III, and the deletion in 11q23 was found in 49% of them. Similar results were obtained by Stilgenbauer et al. (1999), who found that 37 out of 81 MCL samples (46%) had the deletion in 11q23. Nine out of 30 blood samples of CLL/SLL (30%) were found to have the deletion in 11q23, while only 13% (4 out of 32 samples) of the lymph node biopsies of CLL/SLL had the deletion (study II & III). The combined frequency in CLL/SLL is therefore 21% and it is similar to the 20% deletion frequency obtained by others (Döhner *et al.*, 1997). The deletion was found in 24% (4 out of 17) of DLBCL samples, and was absent in FL and HL. For HL, however, we ought to consider that the analysis was not conducted exclusively on the Reed-Stenberg cells that are the malignant cell population in HL (Teerenhovi *et al.*, 1988). The occurrence of the deletion in 11q23 in DLBCL, FL and HL has not been reported before.

The deletion in 11q23 is significantly more frequent in MCL than in any other lymphoma subtypes. We think the deletion in 11q23 could be used as a diagnostic

marker of MCL in addition to the cyclin D1 overexpression. The prognostic value of this abnormality has been shown in CLL (Döhner *et al.*, 1997). Therefore, the 11q23 status is routinely examined using the YAC755b11 probe in the Chromosome and Molecular Cytogenetic Laboratory at the Helsinki University Central Hospital.

About 3% of the CLL/SLL cases progress to DLBCL, which is referred to as the Richter's syndrome (Jaffe *et al.*, 2001). We had three Richter's syndrome cases in our study. One was diagnosed as CLL at the time of the study; clinical follow-up showed that this patient later developed DLBCL. The other two were diagnosed as DLBCL at the time of the study and their records showed that they had previously been CLL patients. Interestingly, all of them had the deletion in 11q23. Although the sample number was small, the current result suggested that the deletion was associated with the development of the Richter's syndrome. CLL patients whose tumor cells have this lesion might be more prone to develop Richter's syndrome and have a poor prognosis. The genes involved in the transformation might reside in this particular region.

CLL and SLL are considered one disease entity in the WHO classification. CLL affects primarily the bone marrow and peripheral blood, and SLL lymph nodes. In our study, we have found that the frequencies of the deletion in 11q23 tend to be different in the blood and lymph node specimens of CLL/SLL, although the difference is not statistically significant. Because of difficulty obtaining the clinical data, we did not know the lymph node involvement status of the patients whose blood samples were tested. Out of the 32 patient whose lymph node samples were tested, 25 patients had the bone marrow infiltration. Although pair-wise comparison is not possible, our results do suggest that the malignant cells with 11q23 deletion may have impaired homing ability to the lymph node and therefore may be more likely to be in the blood circulations. Our hypothesis is supported by a recent study, which demonstrated that functionally relevant adhesion molecules and cell signaling receptors on CLL cells are differentially expressed according to the 11q22.3-q23.1 deletion status (Sembries *et al.*, 1999). Sembries et al. (1999) suggested that the 11q deletion might have impaired several cellular pathways in CLL cells, changing the migratory properties of these cells within lymphoid organs and in peripheral blood. Studies have also shown that typical CLL (Karhu *et al.*, 1997) and typical SLL (Autio *et al.*, 1998) have rather different CGH profiles. All these results urged us to question

whether CLL and SLL are different forms of one disease or if they are actually two distinct disease entities. This matter deserves further investigations.

We also need to bear in mind that CLL itself is a heterogeneous disease, which can be divided into two subgroups based on the Ig V gene mutation and the CD38 expression status. Patients with unmutated Ig V gene and higher percentage of CD38+ cells have poorer prognosis (Damle *et al.*, 1999; Hamblin *et al.*, 1999). On the other hand, 11q23 deletion has been found to define a subset of CLL with poor prognosis (Döhner *et al.*, 1997). It will be interesting to see if the patients with unmutated Ig V gene and higher percentages of Cd38+ cells also have the 11q23 deletion. Or perhaps 11q23 deletion is an independent marker that helps to divide CLL into even more subtypes.

9.5 The role of the *PPP2R1B* gene in MCL and CLL (study IV)

Since it was cloned in 1998, the *PPP2R1B* gene has been found to be mutated in human lung and colon cancer (Wang *et al.*, 1998), but not in hereditary paraganglioma (Baysal *et al.*, 1998), ovarian carcinoma (Campbell & Manolitsas, 1999; Wu *et al.*, 1999) or parathyroid hyperplasia and adenoma (Hemmer *et al.*, 2002). In study IV, we found the deletion of exon 3 of the *PPP2R1B* gene in one CLL sample and the structural rearrangement of this gene in one MCL sample. In both cases, FISH analysis had already revealed deletions in 11q23, which could be considered in accordance with the two-hit theory of tumor suppressor genes.

There were, indeed, some imperfections in our analyses. First, the methylation status of the promoter region was not studied. Second, due to the limited information on the intron sequencies available from the Genebank, none of the PCR primers designed for SSCP analysis allowed the investigation of splice-sites. Third, the Southern-blotting analysis was not complete due to the lack of high quality DNA material. So we cannot exclude the possibility that abnormalities in the *PPP2R1B* gene are in fact more common than is revealed by our study.

However, the mutation frequencies of the *PPP2R1B* gene were too low (2.7% in CLL cases and 2.6% in MCL cases) to explain the frequent deletion in 11q23 in CLL and MCL found by the FISH analyses (30% in CLL cases and 49% in MCL cases). In particular, the RT-PCR analysis carried out on 10 CLL samples revealed only one mutation, while 9 of the 10 samples had the deletion in 11q23. Therefore, we

assume that the role of the *PPP2R1B* gene in the pathogenesis of CLL and MCL is probably minor. There might be another gene or genes in the 11q23 region that play more important roles. The *ATM* gene has been shown to have a pathogenic role in MCL (Stilgenbauer *et al.*, 1999; Schaffner *et al.*, 2000) and CLL (Schaffner *et al.*, 1999). Recently, a retinoid-induced class II tumor suppressor / growth regulatory gene was found to be involved in CLL and located in 11q23 (DiSepio *et al.*, 1998). Its exact location and its role in other types of cancer deserve further investigation. In addition, as was discussed in section 9.2, there might be a yet unknown tumor suppressor gene in the region represented by YAC755b11.

9.6 Marker genes identified by array analysis in MCL (study V)

In study V, we identified eight genes that were differentially deregulated in common and blastoid variant MCL using the regression analysis and the naive Bayes classifier. The most important finding is the possible involvement of the gp130 mediated STAT3 signaling pathway in the MCL blastoid variant transformation. Three genes in the pathway, *CMYC*, *PIM1* and *BCL2* were all up-regulated in the blastoid variant, but not in common MCL. The product of *CMYC* is a major regulator of cell growth and the deregulation of *CMYC* plays a central role in the pathogenesis of lymphoid malignancies as well as other types of cancer [reviewed by (Dang *et al.*, 1999)]. The regulation of *CMYC* is coordinated via multiple signaling pathways and one of them is the gp130-mediated STAT3 signaling pathway (Kiuchi *et al.*, 1999). *PIM1* is frequently up-regulated in human hematopoietic cell lines as well as in tumor cells from leukemia patients (Amason *et al.*, 1989). It is also a target of the gp130-mediated STAT3 signal and cooperates with *CMYC* to promote cell proliferation and to prevent apoptosis. *BCL2* is the downstream target of both *CMYC* and *PIM1* (Shirogane *et al.*, 1999). The simultaneous up-regulation of the *CMYC*, *PIM1* and *BCL2* gene in our study demonstrates the close relationship between them, and suggests that the gp130-mediated STAT3 signaling pathway might be involved in the blastoid variant transformation of MCL.

Abnormalities in *CD45* and *CD70* have been found in other types of lymphoma. Reduced levels of *CD45* expression has been found in CLL patients with poorer prognosis (Sembries *et al.*, 1999). Expression of *CD70* was found in CLL, FL, DLBCL and MCL, although *CD70* was infrequently expressed in normal human B cells *in vivo* (Lens *et al.*, 1999).

Although we are not sure of the mechanism behind the frequent up-regulation of *TOP1* in common MCL, we may be able to utilize this piece of information in clinical trials. It will be interesting to test whether topoisomerase I inhibitors will be effective in the treatment of common MCL.

Marker genes of both common and blastoid variant MCL were identified using the regression analysis and PCA. All but two samples showed up-regulation of *BCL1*, which is in accordance with the immunohistochemistry study. The role of cyclin D1 in MCL is well established and will not be discussed further here.

The annexin II (encoded by *ANX2*) heterotetramer is known to interact with a number of tumor related proteins, e.g. tissue-type plasminogen activator (Cesarman *et al.*, 1994), cystein protease cathepsin B (Mai *et al.*, 2000a), collagen-I and tenascin-C (Chung *et al.*, 1996). The interaction and collaboration between them may enhance tumor cell detachment, invasion and motility (Mai *et al.*, 2000b). The cell surface molecule CD44 has been implicated in tumor metastasis (Sy *et al.*, 1997). It has also been shown that, in mammary epithelial cells, vast majority of CD44 interacts with annexin II in lipid rafts (Oliferenko *et al.*, 1999). Our results further demonstrated the close relationship between annexin II and CD44, and their possible roles as promoters of tumor metastasis.

Regulators of G protein signaling (RGS) inhibit the downstream signaling of the G protein (Kehrl, 1998). They are shown to inhibit the migration and adhesion of lymphoid cells (Bowman *et al.*, 1998; Moratz *et al.*, 2000; Reif & Cyster, 2000). Two members of the RGS family, *RGS1* and *RGS2*, were found to be down-regulated in our study, suggesting a role in preventing tumor cell invasion and metastasis.

The carcinoembryonic antigen CGM1 precursor (CD66d) belongs to the CD66 family, whose members appear to have roles in various biological processes including tumorigenesis (Thompson *et al.*, 1991; Obrink, 1997). One of the family members, CD66a, was shown to act as a tumor suppressor in several cancer models (Skubitz *et al.*, 2000). *CD66d* was found to be down-regulated in our study and it might also have tumor suppressing functions.

We should be careful in interpreting the roles of three genes: T cell surface glycoprotein CD5 precursor gene (*CD5*), T cell differentiation antigen CD6 precursor gene (*TP12/CD6*) and T cell specific transcription factor 1 gene (*TCF7*). We used

purified B cells as the reference, whereas the samples were unpurified lymphoma specimens. The proportion of reactive T cells in mantle cell lymphoma samples may range from 5% to 20%. It is possible that the up-regulation of these three gene was due to the T-cell contamination.

In addition to identify marker genes in common and blastoid variant MCL, the purpose of study V was to look for candidate genes for the proposed tumor suppressor gene in 11q23. No candidate gene was found, partly due to the relatively small number of genes (406) included in the array. However, we should consider this study as a pilot study, preparing us for a larger scale array study in the future.

10 CONCLUDING REMARKS AND PERSPECTIVES

More and more evidences coming from studies by both us and others have shown that the 11q23 region harbors more than one tumor suppressor gene. These genes play important roles in the pathogenesis and progression of a number of different types of cancer, including MCL and CLL. To identify and characterize them will improve our knowledge of these diseases as well as the general knowledge of cancer biology. It is also the starting point of inventing new pharmaceuticals and diagnostic tools. Since we showed that the *PPP2R1B* gene is unlikely to be the tumor suppressor gene we are looking for, attention should be turned to other genes in the area. Besides *ATM* and *TSLC1*, other, as yet unknown, tumor suppressor genes could be found in the critical regions we identified. We could utilize the positional cloning strategy to try to identify them: constructing cosmid contigs over the critical regions; narrowing down the critical regions by FISH using cosmid probes; constructing a CpG island map of the critical regions; sequencing; and so on. On the other hand, the advancement of the array technology might offer better solutions to this problem. We could construct a chromosome or site-specific CGH-matrix array and examine the patient genomic DNA with it. We could also construct a chromosome or site-specific cDNA array and directly study the abnormality in gene expression in the patient material.

The deletion in 11q23 is now widely accepted as a chromosomal abnormality that has diagnostic and prognostic value. It is also evident that CLL patients with this abnormality represent a distinct disease subgroup, with a unique disease progression path and genetic profile. Whether this is true for MCL patients is still unknown and deserves further study. The role of the 11q23 deletion in the development of the Richter's syndrome and its molecular background also deserves more investigation. If a correlation could be established between the 11q23 deletion and the development of the Richter's syndrome, the deletion will be an important prognostic factor. The difference in the frequencies of the 11q23 deletion in lymph node specimens and the peripheral blood specimens in CLL/SLL is also an interesting subject. We ought to look more closely at the genetic profiles of both CLL and SLL. The cDNA array analysis has been used to identify separate disease groups, previously considered as one disease entity (Alizadeh *et al.*, 2000). With careful planning and sample selection, we could also get more insights into the nature of these two diseases by utilizing the array technology.

Not only enormous quantities of biological data, but also new technologies, are emerging with high speed in the fields of cytogenetics, molecular genetics and cell biology. We have gained valuable biological information from our successful utilization of various techniques throughout the studies in this thesis, as well as experience in using the techniques. We hope that this knowledge and experience will be beneficial to our future investigations.

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